Diversity of bone matrix adhesion proteins modulates osteoblast attachment and organization of actin cytoskeleton

Les différents types de molécules d’adhésion modulent l’ancrage des ostéoblastes et l’organisation des fibres d’actine du cytosquelette

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Summary Interaction of cells with extracellular matrix is an essential event for differentiation, proliferation and activity of osteoblasts. In bone, binding of osteoblasts to bone matrix is required to determine specific activities of the cells and to synthesize matrix bone proteins. Integrins are the major cell receptors involved in the cell linkage to matrix proteins such as fibronectin, type I collagen and vitronectin, via the RGD-sequences. In this study, cultures of osteoblast-like cells (Saos-2) were done on coated glass coverslips in various culture conditions: DMEM alone or DMEM supplemented with poly-L-lysine (PL), fetal calf serum (FCS), fibronectin (FN), vitronectin (VN) and type I collagen (Col-I). The aim of the study was to determine the specific effect of these bone matrix proteins on cell adherence and morphology and on the cytoskeleton status. Morphological characteristics of cultured cells were studied using scanning electron microscopy and image analysis. The heterogeneity of cytoskeleton was studied using fractal analysis (skyscrapers and blanket algorithms) after specific preparation of cells to expose the cytoskeleton. FAK and MAPK signaling pathways were studied by western blotting in these various culture conditions. Results demonstrated that cell adhesion was reduced with PL and VN after 240 min. After 60 min of adhesion, cytoskeleton organization was enhanced with FN, VN and Col-I. No difference in FAK phosphorylation was observed but MAPK phosphorylation was modulated by specific adhesion on extracellular proteins. These results indicate that
Introduction

Osteoblasts are the cells responsible for bone formation. They synthesize numerous proteins of the extracellular bone matrix and are involved in the process of mineralization. Osteoblasts are generated from bone marrow osteoprogenitors and several hormones and growth factors contribute to their differentiation and activity. However, interaction of cells with the extracellular matrix is an essential event for differentiation, proliferation and activity of osteoblasts [1]. Osteoblasts are linked to the bone matrix via cell surface receptors (mainly integrin) that bind the specific arg-gly-asp amino-acid sequence (RGD) shared by some glycoproteins of the matrix (thrombospondin, fibronectin, vitronectin, type I collagen, fibrilline and osteopontin, bone sialoprotein [belonging to the SIBLING family: small integrin-binding ligand, N-linked glycoproteins]) [2]. Integrins are $\alpha\beta$ heterodimers with an extracellular domain that binds the RGD sequence, a transmembranous part and a short intracellular sequence linked to cytoskeleton proteins. At least 16 $\alpha$ subunits and 8 $\beta$ subunits have been identified leading to more than 20 different types of integrins. Each type of integrin is able to bind a specific RGD-containing protein but several integrins may also bind the same protein [3]. The major cell vitronectin receptor is $\alpha_4\beta_1$, that can also bind fibronectin (FN); conversely, vitronectin (VN) may also link other integrins. Numerous integrins mediate cell attachment to FN, among them $\alpha_1\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ [4]. All these integrins are expressed at the osteoblast surface [5]. However, in all human osteosarcoma cells some of them may be absent. For example, Saos-2 cells express $\alpha_1\beta_1$, but not $\alpha_2\beta_1$ [6] contrary to MG-63 where only $\alpha_2\beta_1$ (and not $\alpha_3\beta_1$) is present as type I collagen receptor [7].

When bound to their ligands, integrins transmit signals by recruiting cytoskeleton and signaling proteins to sites known as focal adhesions. Formation of focal adhesion provides the physical contact sites of attachment between cells and extracellular matrix. Clustering of integrins in focal adhesion site induces the recruitment and the phosphorylation of tensin and focal adhesion kinase (FAK) that subsequently induces the recruitment of talin, vinculin and $\alpha$-actinin [8,9]. Talin, vinculin and $\alpha$-actinin link the F-actin fibers to the plasma membrane and interact with several proteins such as vaso-dilatator phosphoprotein (VASP) and Arp2/3 proteins which are implicated in the regulation of actin polymerization [10,11]. Rearrangement of F-actin bundles induced by their liaison to the integrin $\beta$ subunit can induce changes in the cell shape [12,13]. This can produce finger-like or sheet-like protrusions (respectively filopodia and lamellipodia) [14].

Synthesis and distribution of cytoskeleton proteins have been involved as a cause or a consequence, in osteoblast morphology, growth, migration, attachment, signaling and functions [15]. The growth of bone cells is associated with changes in organization and synthesis of cytoskeleton proteins and migration implicates dynamic reorganization of the cytoskeleton. Rearrangement and polymerization of actin filaments are a consequence of the linkage between cell surface receptor and bone matrix adhesion protein. As a consequence of the involvement of osteoblast attachment via the integrin-cytoskeleton system, signal transduction events are activated allowing control of gene expression.
in osteoblasts [15]. Orientation of matrix collagen fibers deposited by osteoblasts is controlled by cell orientation, which depends of the cytoskeleton organization. In osteoblasts, actin filaments are abundant and mainly distributed at the periphery of the cytoplasm and in the core of the cell processes [15]. Two other cytoskeleton elements are also present in osteoblasts, microtubules of tubulin (which cross the cytoplasm as continuous structure for long distance between perinuclear space and plasma membrane) and intermediate filaments involved in the nuclear anchorage [16]. Actin is a major cytoskeleton protein that plays a central role in cell shape determination. Changes in the pattern of the actin fibers distribution induce cell morphological changes, particularly in term of cell spreading. It may also determine or reflect functional modifications. A growing consensus of experimental data now suggests that the changes within cytoarchitecture of the cell and activation of specific signal transduction pathways are linked [17].

In this study, osteoblast-like cells from human osteosarcoma (Saos-2) were cultured on FN, VN and collagen type I (Col-I) coated coverslips. Kinetic of cell attachment, morphology and proliferation were studied and the organization of the actin cytoskeleton was determined using light and electron microscopy.

Materials and methods

Materials and reagents

Human osteoblast-like cells, Saos-2, derived from osteogenic osteosarcoma (HTB 85) were purchased from ATCC (Manassas, USA). Dulbecco’s Modified Eagle’s Medium (DMEM), L-glutamine, penicillin/streptomycin and trypsin/EDTA were all from Biomedia (Boussens, France) and fetal calf serum (FCS) from Polylabo (Strasbourg, France). Poly-L-lysine (PL), human fibronectin (F 0895), calf collagen type I (C9791), hexamethyldisilazane (HMDS), 3,3’ diethios bis (propionic acid N-D oxysuccinimide ester) (DTSP), Triton X-100, EGTA, Piperrazine-N, N’-bis 2-ethanesulfonic acid (Pipes), Dnase I (D 7291), Rnase A (R 6513), Phalloidin-TRITC (P1951), paraformaldehyde, sodium borohydride, rabbit polyclonal antibody against-actin (A 2066), rabbit polyclonal antibody against—focal adhesion kinase (F-2918) were purchased from Aldrich-Sigma (Saint Quentin Fallavier, France). The mouse monoclonal antibody against phospho-p44/42 MAPK was from Ozyme (Saint-Quentin-en-Yvelines, France). Chloroform was from Aldrich. Human VN (12165064) and 6-well plates came from Life Technology (Eragny, France). Bovine serum albumin (BSA) was from Euromedex (Sofffelwyershen, France), PEG 6000 and glutaraldehyde from Merck (Fontenay-sous-bois, France). Glass coverslips were from CML (Nemours, France), and 100 and 250 mm plastic dishes from Fisher Scientific (Elancourt, France). DNA quantification was done on a Shimatsu spectrometer (Kyoto, Japan). Carbon-sputtering was done with a MED 020, Bal-Tec (Chatillon sur Cher, France). The scanning electron microscopy (SEM) was done using a JEOl JSM-6301 F from JEOL (Paris, France) and the fluorescent microscopy with an Olympus Fluoview confocal microscope (Paris, France).

Cell culture

Saos-2 were routinely grown on 250 mm plastic dishes in DMEM supplemented with 10% FCS, 4 mm L-glutamine and 50 U/ml penicillin/streptomycin, at 37 °C in humidified and 5% CO2 atmosphere. At confluence and before experiments, cells were FCS-deprived overnight at 37 °C with 5% CO2 in a humidified atmosphere and then passaged with 0.1% trypsin/EDTA. For experiments, the supports of culture (coverslips or plastic dishes) were coated according to the followed conditions:

- DMEM medium culture alone;
- DMEM with 0.001% PL as unspecific substrate;
- DMEM supplemented with 10% FCS;
- DMEM supplemented with 2 μg/ml FN;
- DMEM supplemented with 1 μg/ml VN;
- DMEM supplemented with 10 μg/ml type I collagen.

For each condition, 100 mm plastic dishes and 25 mm glass coverslips were sterilized by ultraviolet irradiation for 2 h and then coated by pre-incubation at 4 °C, overnight, just before experiment. The coating medium was removed and cells were plated on coated glass coverslips at 2.5 ×10^5 cells per coverslip and at 2 ×10^5 per cm² in 100 mm plastic dishes. For all the following experiments, cultures were done in DMEM alone without any supplementation.

Kinetic of cell adherence

For each cultured condition, cells were seeded and cultivated in triplicate onto coated glass coverslips (2.5 ×10^5 cells/coverslip) for 5, 30, 60, 120 and 240 min at 37 °C in humidified and 5% CO2 atmosphere. Before harvesting, cultures were rinsed in PBS and then treated with 0.1% trypsin/EDTA. The number of cells was counted using a Malassez cell and results were expressed as percentage of adherent cells vs. seeded cells.

For DNA content measurements, cell cultures were rinsed in PBS and lysed in potassium hydroxide 0.6 N by 3 cycles +37 °C—196 °C. Suspensions were incubated 1 h at 37 °C to hydrolyze RNA. Hydrogen perchorlides was then added at the final concentration of 0.6 N and incubated 1 h at 37 °C to precipitate total DNA and proteins. After centrifugation 10 min at 30,000 g, pellets were solubilized in hydrogen perchorlides 0.6 N and incubated 10 min at 80 °C for DNA hydrolyze and then 1 h at 4 °C. After centrifugation, proteins are pelleted and the DNA in the supernatants was quantified by dosage at 260 nm using a spectrometer. Results were expressed in μg of DNA per coverslip.

Actin visualization with TRITC-labelled phalloidin

Cells were cultured onto coated coverslips (2.5 ×10^5 cells/coverslip) for 5, 30, 60, 120, 240 min and 24 h. After washing in PBS, cells were fixed with 4% paraformaldehyde in PBS for 20 min, at room temperature. Following extensive rinsing with PBS, cells were treated in the dark with 12.7 μM TRITC-labeled phalloidin in PBS, for 30 min. The actin cytoskeleton was identified under confocal microscope with a highpass BA610IF filter.
Scanning electron microscopy (SEM)

For morphological study and quantitative measurements, cells were plated onto coated glass coverslips (2.5 × 10^4 cells/coverslip) and cultured for 5, 30, 60, 120, 240 min and 24 h. Coverslips were fixed in 2.5% glutaraldehyde in PBS, dehydrated in graded ethanol series, desiccated in hexamethyldisilazane and air dried. Samples were carbon-coated prior SEM examination at 5 kV. Analysis and measurements of cell surface area were performed on at least 10 cells, from each culture condition and time, by image analysis using SEM photographs at the original magnification of ×1500 and NIH Scion image for Windows.

For cytoskeleton examination, cells were plated onto coated glass coverslips (2.5 × 10^4 cells/coverslip), cultured for 5, 30, 60, 120, 240 min and 24 h and prepared according to the method of Bell et al. [18–20]. Briefly, cells were pre-fixed during 5 min at 37 °C in 0.02% DTSP in PBS. The cytoskeleton was then prepared by extracting cells with a non-ionic detergent, which solubilized the membrane and revealed the cytoskeleton. Coverslips were treated for 5 min at 37 °C, with 1% Triton X-100 and 0.02% DTSP in Micro Tubule Stabilizing Buffer, MTSB (MTSB: EGTA 1 mM, PEG 6000 4% and Pipes 100 mM, at pH 6.9 in deionised water), and then treated twice with 1% Triton X-100 in MTSB, without DTSP, for 5 min each, at room temperature. Cells were then incubated in 100U/ml Dnase I and 100 μg/ml Rnase A in MTSB, 30 min at 37 °C. Coverslips were post-fixed in 2% paraformaldehyde with 0.1% glutaraldehyde in PBS for 5 min, rinsed 3 times in PBS and treated by sodium borohydride for 5 min at room temperature to block aldehyde sites. For SEM examination of the cytoskeleton, cells were then treated as for morphological study.

Proteins of the cytoskeleton (actin, tubulin and vimentin) were identified using immunogold labeling. After sodium borohydride incubation, cells were treated with 5% BSA in PBS to block non-specific antigenic sites and rinsed in PBS with 0.1% BSA. Cells were incubated for 2 h at 37 °C with the following first antibodies diluted at 1/100 in 0.1% BSA in PBS: goat anti human actin, rabbit anti human tubulin or goat anti human vimentin. Protein A conjugated with 20 nm colloidal gold beads, diluted at 1/100 in PBS with 0.1% BSA, was applied overnight at 4 °C. The cultured coverslips were then treated as for SEM examination.

Image analysis

Two-texture analysis were developed in VisualBasic (Microsoft) and used sequentially on the SEM images of the cytoskeleton.

The "blanket" fractal analysis [21]

We calculated the fractal dimension by using dilatation and erosion of an image, the mathematical details have been presented elsewhere [22]. The structural element used was a cross rod-shaped element. Given this structuring element of size ε, a dilation and an erosion of the image provided two new covering images: respectively, the upper and the lower ones. The volume of the blanket, i.e., the volume enclosed between the dilatation and erosion images, was measured. The number of dilations and erosions ranged from 1 to 10 and the volume as measured each time. The fractal dimension $D_{\text{blank}}$ was computed by plotting the logarithm of the volume against the logarithm of ε and searching the slope coefficient by the least-squares method.

The "skyscrapers" fractal analysis [23]

Pixels that constituted an image were considered as skyscrapers whose height was represented by the grey level. The roof of the skyscrapers was a square of size ε. The surface area of the image was obtained by measuring the sum of top surfaces and sum of the exposed lateral sides of the skyscrapers. Gray levels of adjacent pixels were then averaged in squares of ε: 2, 4, 8, 16, and 32 pixels to produce new images; the surface area of each image was calculated for each ε. The fractal dimension of the surface was determined by plotting the logarithm of the surface against the logarithm log ε. A linear regression line was computed only on the aligned points by the least-squares method. The fractal dimension was obtained as $D_{\text{sky}} = 2 + $ slope [22].

Immunoprecipitation and western blot analysis

Cells were plated onto 100-mm coated plastic Petri dishes (2 × 10^5 per cm²) for 5, 30, 60 and 120 min at 37 °C in...
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Figure 2  Scanning electron microscopy of the effects of matrix proteins on the morphological aspect of cells cultured during 5, 30, 60 min and 24 h. Coverslips coated with poly-L-lysine (PL), DMEM alone, fetal calf serum (FCS), fibronectin (FN), vitronectin (VN) and type I collagen (Col-I). Cell spreading increased with time, excepted on PL.  

Microscopie électronique à balayage des effets des protéines matricielles sur l’aspect morphologique des cellules cultivées pendant 5, 30, 60 minutes et 24 heures. Lamelles couvre-objet revêtues de poly-L-lysine (PL), DMEM seul, sérum de veau fœtal (FCS), fibronectine (FN), vitronectine (VN) et collagène de type I (Col-I). L’étalonnage cellulaire a augmenté avec le temps, à l’exception de la PL.

Cells were then solubilized in 1 ml of lysis buffer (50 mM Tris pH 7, 100 mM NaCl, 50 mM NaF, 3 mM Na3PO4, 2 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/µl pepstatin, 1 mM PMSF, 1% NP40) for 1 h at 4 °C. The lysate was centrifuged for 30 seconds at 4 °C at 12,000 rpm and the lysate protein concentrations were determined by a colorimetric assay (Bio-Rad Laboratories, Hercules, California) and standardized prior to further analysis.

For MAPK analysis, 15 µl of sample buffer (1 M Tris, 0.025% bromophenol blue, 10% SDS, 10% (v/v) glycerol, 5% mercaptopropanediol) was added to 25 µl of lysate. After boiling 10 min at 100 °C, cell lysates were ready to western blot analysis. For FAK analysis, cell lysates must previously undergo immunoprecipitation. For immunoprecipitation, cell lysates were incubated with primary antibody against focal adhesion kinase overnight at 4 °C with rocking, followed by incubation with protein A-sepharose for an additional 2 h at 4 °C. Protein A-sepharose complexes were pelleted and washed 2 times with lysis buffer, one time with lysis buffer without detergent and one time with Tris 50 mM. Bound proteins were eluted in 40 µl sample buffer (1 M Tris, 0.025% bromophenol blue 10% SDS, 10% (v/v) glycerol, 5% mercaptopropanediol) by boiling 10 min at 100 °C.

Proteins in the sample buffer were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gels for FAK analysis and on a 10% SDS-polyacrylamide gels for MAPK analysis. Proteins were electrophoretically transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). Blots were blocked with 6% BSA for FAK and 5% milk for MAPK in 200 mM NaCl and 50 mM Tris pH 7.6 overnight at 4 °C, and subsequently incubated with primary antibody (0.5 µg/ml) in 1% BSA/200 mM NaCl/50 mM Tris pH 7.6/0.05% Tween 20, for 5 h at room temperature. Membranes were then washed briefly and incubated with goat anti-mouse IgG or goat anti-rabbit IgG peroxidase conjugates
for 1 h. Following extensive washing, immunoreactivity was detected on film using chemiluminescent immunodetection (ECL kit, Amersham Corp. Arlington Heights, IL). Images from ECL autoradiograms were captured using ImageJ (NIH image software).

Statistical analysis

Statistical analysis was done using Systat software, release 6.0.1 (SPSS, Chicago, Ltd) with a nonparametric analysis of variance (ANOVA) with Bonferroni’s probability least significant difference post-hoc test. All data were expressed as mean and standard deviation. Differences were considered significant when $P < 0.05$.

Results

Cell attachment assays

The number of adherent cells onto the different coated coverslips was evaluated by counting with the Malassez cell (result no shown) and the dosage of DNA content (Fig. 1). Results were similar with the two methods. For each type of coated coverslips, cell adherence was time dependent. The comparison at each time did not show any important differences until 60 min. After 60 min, cell adherence was significantly decreased on VN and PL as compared with other conditions. At 240 min, adherence on VN was decreased compared to FCS, FN and Col-I and adherence on PL was diminished versus on FN or Col-I. There were no significant differences for other coatings.

Morphological aspects

Numerous adherent cells were observed in SEM as soon as from 5 min onto coated supports. However on FCS-coated coverslips scarcely distributed cells were observed (Fig. 2). Cells were small and globulous until 30 min on DMEM, FCS, FN, VN and Col-I but larger on PL. After 1 h, very few cells were observed on FCS coatings. Cells appeared rounded but some flat cells were presents on VN, FN and Col-I. On PL, cells were numerous, large and very flat. At 4 h (results not shown) and 24 h of adhesion, cells cultured on PL showed a globular appearance and on the other coatings, large cells were in majority (Fig. 2).

Cell surfaces were measured by image analysis (Fig. 3). After 30 min, cells cultivated on the PL coating were significantly larger than on all other supports. However, their surface significantly decreased with time while the surface of cells cultivated on all others coated supports significantly increased with time. After 24 h of culture, the cell surface area was significantly increased on FN vs. all other supports.

After treatment with TRITC-phalloidin, the actin network was identified in confocal microscopy as red filaments (Fig. 4). After 5 min of adhesion, cells were round in all culture conditions but no organized cytoskeleton network was observed. Actin filaments were observed first in cells cultivated on VN, FN and Col-I from 1 h of culture. Cells on VN showed actin filaments mainly distributed in periphery while the actin filaments were observed throughout the cytoplasm on FN and Col-I. At 24 h, flat cells were present on FCS, FN, VN and Col-I with an important network of actin fibers. On PL and DMEM, cells are smaller, rounder and no actin fibers were observed (Fig. 4).
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**Figure 4** Effects of matrix proteins on actin cytoskeleton analyzed by confocal microscopy. Cells were grown during 5, 60 min and 24 h. DMEM alone, and onto coverslips coated with poly-L-lysine (PL), fetal calf serum (FCS), fibronectin (FN), vitronectin (VN) and type I collagen (Col-I). Clearly organized actin cytoskeleton was detected with FCS at 24 h, FN, VN and Col-I from 60 min.

**Effets des protéines matricielles sur les fibres d’actine du cytosquelette : analyse par microscopie confocale. Les cellules ont été cultivées pendant 5, 60 minutes et 24 heures. DMEM seul, et sur des lamelles recouvertes de poly-L-lysine (PL), sérum de veau fœtal (FCS), fibronectine (FN), vitronec- tine (VN) et collagène de type I (Col-I). Les fibres d’actine du cytosquelette sont clairement détectées avec le FCS à 24 heures, FN, VN et Col-I à partir de 60 minutes.**

After extraction of the plasma membrane and immunogold labeling, elements of the cytoskeleton were observed: actin microfilaments, microtubules of α- and β-tubulin and vimentin intermediate filaments (Fig. 5). On FCS, cells appeared very small and round until 240 min so no cytoskeleton could be analyzed on such samples. Two regions in the cell cytoskeleton were observed: one perinuclear and one at the cell periphery. The perinuclear cytoskeleton did not show any differences whatever time of culture and coated support and was mainly represented by vimentin as identified by immunogold (Fig. 5a). After 1 h, the cytoskeleton at the periphery of the cell showed different aspects of organization according to the coating used. Immunogold labeling identified actin (Fig. 5b), fibronectin (Fig. 5c), and tubulin (Fig. 5d) but it was not possible to characterize each structure of the cytoskeleton so it was not possible to determine the degree of organization of each element of the cytoskeleton (Figs. 5 and 6). On PL and FCS, cytoskeleton appeared as a compact mesh with no individualized filament, no filipodia or lamellipodia. On DMEM, the cytoskeleton was not really developed, but cells send long filopodia towards other cells. These filopodia contained a single and very long filament. On VN, the filaments seemed to be much more polymerized and oriented parallel to the cell profile. On FN and Col-I, the cytoskeleton seemed to be much more oriented; individuals filaments were packaged together in large bundles extending throughout the cell (Figs. 5 and 6).

Texture analysis provided different results according to the algorithm used. $D_{\text{bench}}$ showed few differences between coatings and times. The heterogeneity of the cytoskeleton was significantly different between 5 min and 24 h on PL and Col-I ($P < 0.05$). At 24 h VN heterogeneity was significantly lower than on cells grown on PL or FCS ($P < 0.05$). $D_{\text{sky}}$ analysis revealed much more differences. The $D_{\text{sky}}$ dimension, reflecting cytoskeleton heterogeneity was significantly higher at 24 h than at 5 min for PL, DMEM, FN and Col-I ($P < 0.05$). No differences were observed with FCS and VN at any time. $D_{\text{sky}}$ measured on VN was lower than on DMEM and FCS at 240 min and 24 h and also lower than on PL, FN and Col-I at 24 h ($P < 0.05$).

**Western blot analysis**

FAK phosphorylation (Fig. 7) showed no significant difference between culture conditions at all times. However, a weaker expression was observed on PL and DMEM until 60 min. For each cultured condition, FAK phosphorylation seemed to grow up with time.

MAPK phosphorylation (Fig. 8) was lower at each times in cells plated on PL until 60 min. In cells cultured with DMEM, FCS and Col-I, MAPK phosphorylation showed no significant difference with time. MAPK phosphorylation decreased with time in cells plated on FN and VN.

**Discussion**

In this study, the influence of extracellular proteins on osteoblastic adhesion and shape was evaluated. Osteoblasts synthesize numerous extracellular proteins including type I collagen, the principal organic component of bone matrix and a wide variety of non-collagen proteins such as...
fibronectin, osteopontin, and bone sialoprotein. Adhesive interactions between cells and their substrata, mediated by cell surface integrin and extracellular matrix proteins, appear to result in massive rearrangement of the cell cytoskeleton. These early events promote several structural changes such as cell spreading and initiate signaling cascades, which modulate genes expression [24,25].

This study strongly supported the hypothesis that the organization of the cytoskeleton was mainly related to the extracellular matrix protein on which osteoblast adhered. The kinetics of osteoblast-cell adherence and the aspects of the cell shape were dependent of the nature of the substrate without any influence of cell density which was similar after long time culture whatever matrix protein used was [26]. This is in agreement with reports showing different adhesive and spreading kinetics of cell cultured on FN, Col-I or serum-free medium [27–29]. Quantification of the SEM images of the cytoskeleton was only possible by using techniques developed to evaluate the complexity of an image.

Fractal methods for measuring the texture of an image are useful and can provide more information than those based on Euclidean geometry. It is also well known that the fractal algorithms should be adapted to the texture to measure. In the present study, the skyscraper algorithm provided more information than the blanket because there was no preferential distribution of the cytoskeleton fibers [30,31].

Cells cultured on purified adhesion proteins that contain RGD-sequences had the highest surface area and only cells on PL showed a decrease with time in cell surface area. The cell rounding on PL might be related to disassociation of cell receptor-substratum complexes, which causes a dissipation of isotonic tension at cell surface and retraction of cortical microfilaments. This might lead to a growth arrest.

The cellular attachment in serum-free medium indicates that osteoblast binding can occur in the absence of serum or exogenously added cell adhesion proteins. When cells adhere to glass, they use presumably other mechanisms or non-integrin receptors to mediate cell attachment. Our

Figure 5 Immunogold labeling of cytoskeleton proteins for (A) perinuclear vimentin, (B) actin, (C) vimentin intermediate filaments, (D) tubulin microtubules, (E) negative control for vimentin and (F): negative control for actin. White arrows indicate some of 20 nm gold nanoparticles and arrowhead points out to the nucleus.

Immuno-marquage des protéines du cytosquelette (A) vimentine péri-nucléaire, (B) l'actine, (C) filaments intermédiaires vimentine, (D) microtubules de tubuline, (E) contrôle négatif pour la vimentine et (F) contrôle négatif pour l'actine. Les flèches blanches indiquent des nanoparticules d'or de 20 nm et les têtes de flèche pointent vers le noyau.
results confirm previous observations, which did not report marked changes in attachment in presence or absence of a specific or preferred ligand such as fibronectin [32]. For other matrix proteins (FN, VN or Col-I), the osteoblast-cell had a relatively flat shape. These findings demonstrate the specific role of extra cellular matrix molecules to the cell binding, the actin polymerization triggering, the contact adhesion formation and the stabilization of cell shape [16,33].

Previous authors suggested that fibronectin promoted both osteoblast attachment and spreading [34] and that plating on matrices such as type I collagen and fibronectin accelerated osteoblastic cell maturation [35–37]. Moreover, FN functions as a survival factor for mature osteoblasts [38].

**Figure 6** Effects of matrix proteins on ultrastructural cytoskeleton aspect. Cells were cultivated in DMEM alone for 5, 60 min and 24 h onto coverslips coated with poly-L-lysine (PL), DMEM, fetal calf serum (FCS), fibronectin (FN), vitronectin (VN) and type I collagen (Col-I).

**Effets des protéines matricielles sur l’aspect ultrastructural du cytosquelette.** Les cellules ont été cultivées dans du milieu DMEM seul pendant 5, 60 minutes et 24 heures sur des lamelles recouvertes de poly-L-lysine (PL), DMEM, sérum de veau fœtal (FCS), fibronectine (FN), vitronectine (VN) et collagène de type I (Col-I).
Figure 7 Effects of matrix proteins on FAK phosphorylation. Cells were grown in DMEM alone during 5, 30, 60 and 120 min onto coverslips coated with poly-L-lysine (PL), DMEM, fetal calf serum (FCS), fibronectin (FN), vitronectin (VN) and type I collagen (Col-I) and FAK phosphorylation study was performed by western blot.

Effects des protéines matricielles sur la phosphorylation de FAK. Les cellules ont été cultivées dans du DMEM seul pendant 5, 30, 60 et 120 minutes sur des lamelles recouvertes de poly-L-lysine (PL), DMEM, sérum de veau fetal (FCS), fibronectine (FN), vitronectine (VN) et collagène de type I (Col-I) et l'étude de la phosphorylation de FAK a été réalisée par western blot.

Osteoblastic cells cultured on a collagen substratum adhered and spread over it. Col-I, like FN anchorage is important for osteogenesis but may function at different stages of the osteoblast differentiation program [39]. The attachment of osteoblasts to the collagenous matrix had important effects on the expression of the osteoblast phenotype and osteoblast marker genes such as alkaline phosphatase and osteocalcin [40]. In cell cultivated on VN, polymerized actin fibers are observed but they are mainly at the periphery of the cell. Some authors supposed that, in epithelial cells, this circumferential band of actin filaments provided the structural support for cell-cell junctions [41].

Results reported in this study seem to confirm that integrins convey information from the extracellular environment by linkage between the extracellular matrix and actin cytoskeleton. They transmit signals by organizing the cytoskeleton and regulating cell shape [42,43]. Such changes in cell shape and cytoskeleton organization might regulate gene expression and cell biosynthesis [44,45] and thereby control the differentiation and growth of cells [46]. Current speculation postulates that the formation of large aggregates of signal transduction molecules on a cytoskeleton framework provides high local concentrations of receptors, enzymes, substrates, and structural molecules. This protein clustering facilitates activation of signaling pathways by stimulating tyrosine phosphorylation cascades linked to MAPK and other pathways [47] and organization of adhesive sites. Adhesion on various matrix proteins induces structural changes in cells, and may alter the conformation and interactions of integrin receptor thereby leading to activation of different signal transduction pathways, thus would be activated by cell adhesion or receptor occupancy. Finally it may be necessary that the integrin receptors could be engaged with an extracellular ligand in order to mediate signal transduction. Such a result is clearly consistent with the unique dependency of mechanotransduction on the maintenance of an intact cytoskeleton [32].

Binding of integrin and the subsequent cell adherence stimulate the formation of membrane-associated focal adhesion complexes [32]. Some authors have reported that FAK might play a role in the regulation of MAPK in inducing tyrosine phosphorylation of p44MAPK in NIH 3T3. So, MAPK activity may be related to cell shape and cytoskeleton organization [48]. In this study FAK and MAPK are phosphorylated after cell adhesion. If no significant differences were observed for FAK phosphorylation, the MAPK phosphorylation differs with time and type of matrices. These data suggested that FAK and MAPK phosphorylations are part of regulation in cytoskeleton organization but other cell signaling pathways might be involved. That is in agreement with findings showing that Rho pathway was implicated in integrin-mediated focal adhesion formation and may also enhance actin polymerization [49].

In this study the influence of matrix proteins on cell attachment and cytoskeleton organization was investigated. These results show that binding to different adhesion
proteins induces changes in cytoskeleton organization. This suggests that cells are able to distinguish specific integrin-ligand binding, to induce different intracellular pathways in response and in consequence to modulate cytoskeleton organization.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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References


