Volume 222, number 2, 311–316 FEB 05181 October 1987

Cytoskeletal protein synthesis and organization in cultured mouse osteoblastic cells

Effects of cell density

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Received 13 August 1987

The most abundant cytoskeletal proteins synthesized in mouse endosteal osteoblastic cells were identified employing two-dimensional polyacrylamide gel electrophoresis and immunoblotting. The relative rate of synthesis of the proteins were measured on radioautograms of detergent-soluble and -insoluble lysates of the cells labeled with [^{35}S]methionine. Doubling initial cell density induced a 10–45% reduction in the de novo synthesis of actin, α-actinin, vimentin and β-tubulins with no change in α-tubulins. Increasing cell density caused a 45% decrease in the polymerized form of actin with no change in the unpolymerized fraction, suggesting a correlation of alteration of the organization and synthesis of proteins.

Cytoskeleton; Actin; Vimentin; Cell density; Tubulin; (Mouse bone cell)

1. INTRODUCTION

A number of studies have indicated that cell shape and cell-cell contacts play a major role in the regulation of cell proliferation and differentiation [1,2]. Cell configuration and cell-to-cell contact inhibition of growth affect the organization of the cytoskeleton and changes in gene expression [3]. It is therefore of interest to determine whether the changes in cell shape with cell density are associated with variations in the expression of genes encoding for cytoskeletal components, a phenomenon which may control cell growth and differentiation [3,4].

The normal process of bone formation involves the interaction of osteoblasts with the extracellular collagen matrix that these cells synthesize. A clear relationship between osteoblastic cell shape and collagen fiber orientation has been described both in vivo and in vitro [5,6], a phenomenon that may involve both the organization and synthesis of the cytoskeleton. Ultrastructural and immunofluorescence studies have identified some cytoskeletal elements in osteoblastic cells [7,8]. Disorganization of the cytoskeleton in these bone cells has been shown to interfere with the secretory process of extracellular proteins [9]. Moreover, alteration of osteoblast function by calciotropic hormones is associated with changes in cell shape and cytoskeletal organization [10]. Because of the possible involvement of the cytoskeleton in control of osteoblast function, we sought to identify the expression of cytoskeletal proteins in osteoblasts and to determine the changes in organization and biosynthesis of these components in relation with cell density. Studies of the cytoskeletal proteins in bone cells have been generally limited to changes in the organization of these elements [5–11]. We describe here for the first time the identification of cytoskeletal proteins in osteoblastic cells in two-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

dimensional (2D) gel patterns. The synthesis of the most abundant proteins were analyzed in normal endosteal osteoblastic cells isolated from mouse caudal vertebrae [12]. The cytoskeletal structures were separated and identified by use of high-resolution 2D gel electrophoresis. The measurement of the relative rate of synthesis of the main cytoskeletal proteins showed that the syntheses of polymerized actin and α-actinin were elevated in osteoblastic cells plated at low density. Increasing cell-cell contacts resulted in a reduction in the biosynthesis of insoluble actin, α-actinin, vimentin and β-tubulins. In addition increasing cell density was associated with marked changes in cytoskeletal organization, and occurrence of vimentin cleavage products.

2. MATERIALS AND METHODS

2.1. *Cell isolation procedure*

Mouse osteoblastic cells were isolated from the endosteal trabecular area of vertebrae. The method of isolation and culture was described in [12]. Briefly, caudal vertebrae from 10-day-old mice were dissected and freed of periosteum and bone marrow. Endosteal osteoblasts were isolated by migration on a nylon mesh and cultured in DMEM (Flow) + 10% fetal calf serum. The isolated cells show in great majority (>90%) morphologic and functional characteristics of osteoblasts such as high alkaline phosphatase activity, response to 1,25-dihydroxyvitamin D_3, stimulation of adenylate cyclase in response to parathyroid hormone, in vitro production of type I collagen and matrix mineralization [12].

2.2. *Cell cultures and labeling*

Osteoblastic cells obtained from primary cultures were plated at 4000 or 8000 cells/cm^2 in 60 mm plastic petri dishes (Falcon). The medium was changed every 2 days and the cells were cultured for 5 days. At this stage, the cells were at subconfluence or at confluence according to their respective initial cell density. On the last day of culture, the cells were labeled with L-[^{35}S]methionine (74 MBq/mmol, Amersham, 25 μCi/ml medium). After 24 h, the cells were rinsed 3 times in calcium-free phosphate buffer solution (PBS), collected into buffer A (0.1 M 2-(N-morpholino)ethanol sulfonic acid, pH 6.4, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1.15 mM β-mercaptoethanol) plus 0.2 M GTP, α_2-macroglobulin and 84 mM leupeptin. The cells were then lysed by addition of 1% Triton X-100 (Sigma) and harvested. The supernatants and pellets were centrifuged (10000 × g, 10 min, 4°C), resuspended in buffer B (16.6% β-mercaptoethanol, 6.64% (w/v) Nonidet P-40) and immediately treated for isoelectric focusing [13].

Radioactivity was determined in aliquots of supernatants (3 μl) and pellets (2 μl) by liquid scintillation counting after precipitation in 7.5% trichloroacetic acid.

2.3. *2D gel electrophoresis*

The detergent-soluble and -insoluble fractions were subjected to 2D SDS gel electrophoresis according to O'Farrell [13] with some modifications [14]. The pH gradient (pH 4.5–7.25) was measured by cutting the isoelectric focusing gel into 5-mm sections which were placed individually in 2 ml degassed H_2O and shaken for 10 min at 37°C, the pH being determined using a pH meter. The SDS electrophoresis gels were calibrated using standard proteins of known molecular mass [carbonic anhydrase, 31 kDa; ovalbumin, 45 kDa; BSA, 66.2 kDa; phosphorylase b, 92.5 kDa (Biorad)]. The gels were run concurrently (4 samples per cell extract). The gels were stained with Coomassie brilliant blue, dried and exposed to Kodak Industrex AX film. The protein patterns were compared on stained gels and gel autoradiographs to check the reproducibility between runs of the same cell extract and of separate extracts.

2.4. *Immunoblotting procedure*

For immunoblotting, the proteins separated on the 2D gels were electrophoretically transferred to nitrocellulose membranes as described by Towbin et al. [15]. The transfer was carried out at 300 mA for 3 h. After blotting, the nitrocellulose sheets were equilibrated for 1 h at 37°C in PBS plus 3% BSA, then in PBS plus BSA (0.5%) plus 0.2% Tween 20, followed by 18 h incubation at room temperature with a 1/2 dilution of either a monoclonal antibody which reacts with all intermediate filaments [16] or with a rabbit polyclonal antibody to α-actinin [17]. The sheets were washed and reacted with a 1/200 dilution of peroxidase-labeled mouse antiserum for 2 h. After

rinsing, the sheets were revealed in 2 M Tris-HCl (pH 7.6), 0.03% H_2O_2 and 1.4 mM 3,3'-diaminobenzidine and dried.

2.5. *Quantification of labelled proteins*

After resolution and identification of labeled proteins on 2D gels, the protein spots containing actin, vimentin, α-actinin and tubulins were excised from the gels, placed in H_2O_2 at 60°C and the proteins were dissolved in a Beckman tissue solubilizer (BTS 450) at 40°C for 30 min. The radioactivity was then measured in a liquid scintillation counter. The quantification of [^{35}S]methionine incorporated into the different proteins was calculated from the average values obtained from two different runs of the same samples. The [^{35}S]methionine incorporation into the detergent-soluble and -insoluble proteins was expressed as the ratio over the total radioactivity recovered in the soluble or insoluble fractions. It was also expressed relative to the total radioactivity in the whole initial cell lysates.

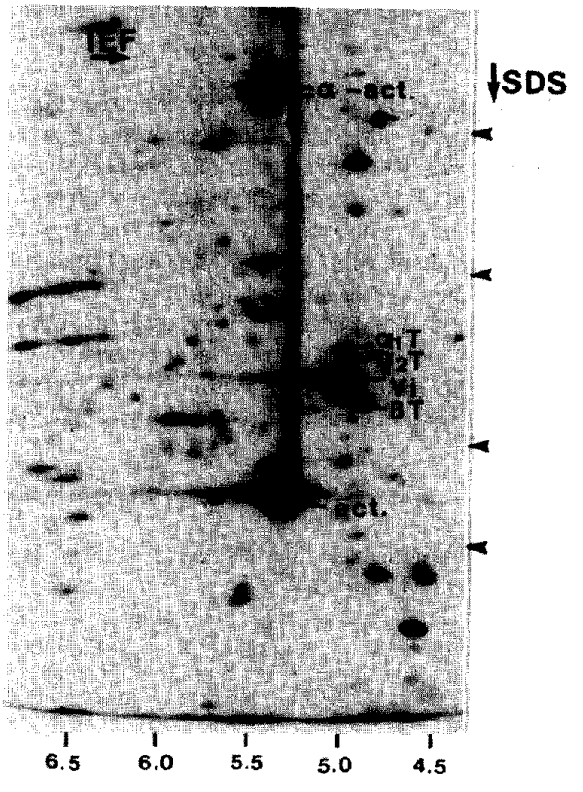

Fig.1. Radioautogram of 2D gel pattern of detergent-insoluble lysate of mouse osteoblastic cells. Cells were labeled for 24 h with [^{35}S]methionine, lysed and the insoluble proteins recovered in the pellet were treated for electrofocusing. Arrowheads indicate positions of molecular mass markers: 92.5, 66.2, 45 and 31 kDa, respectively. α_1, α_2T, βT, α- and β-isotubulins; Vi, vimentin; act., actin; α-act., α-actinin.

3. RESULTS

3.1. *Identification of protein spots*

The protein spots in mouse osteoblastic cells were identified by migration according to their isoelectric point and molecular mass in 2D gel electrophoresis (figs 1,2). Actin and α- and β-tubulins were clearly resolved and were found in both gels run with detergent-soluble or -insoluble proteins. Vimentin was found only in the pellet (detergent-

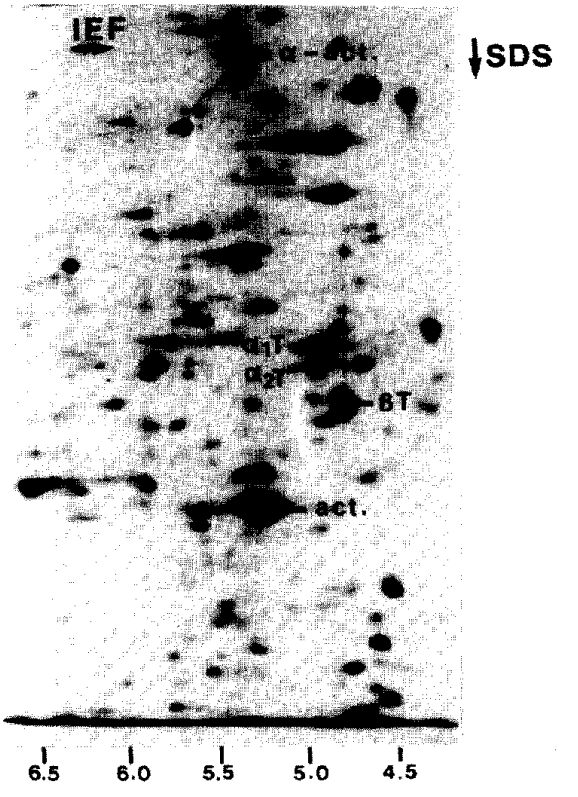

Fig.2. Radioautogram of 2D gel pattern of detergent-soluble lysate of mouse osteoblastic cells. Soluble proteins recovered in the supernatant were treated for electrofocusing. Proteins and protein markers are identified as in fig.1.

insoluble) extract (fig.1) and was further identified after staining with a monoclonal antibody recognizing all classes of intermediate filaments (fig.3A). This monoclonal anti-vimentin antibody also recognized cleavage products of vimentin which were more clearly apparent at the higher cell density. α-Actinin migrated as a well-resolved dimer, predominantly in the insoluble fraction (fig.1) and was further identified by immunoreaction with a specific antibody (fig.3B).

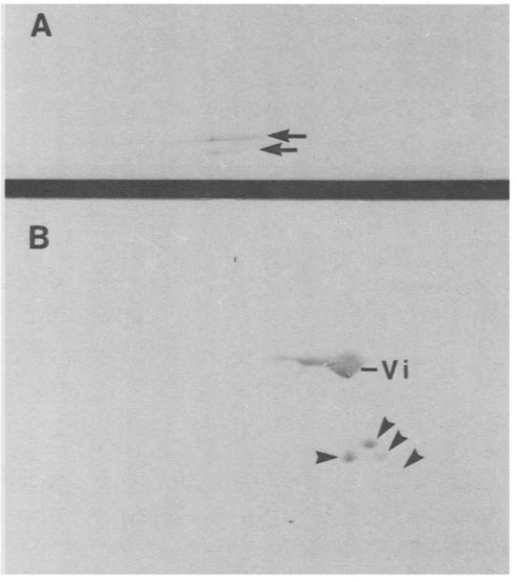

Fig.3. Identification of α-actinin (A) and vimentin (B) by immunoblotting and staining with polyclonal and monoclonal antibodies, respectively. α-Actinin is well-resolved as a dimer (arrows). Vi, vimentin; arrowheads point to vimentin cleavage products occurring in dense cell culture and also recognized by the intermediate filament antibody.

3.2. *Changes in cytoskeletal protein synthesis with cell density*

The relative rate of synthesis of the major cytoskeletal proteins was determined at two cell densities. Doubling the initial cell density resulted in a slight (10%) reduction in total [^{35}S]-methionine-labeled actin recovered in whole cell lysates (table 1). Similarly, vimentin and β-tubulins showed a total rate of synthesis which decreased by about 14% whereas that of α-tubulins remained unchanged.

3.3. *Changes in cytoskeletal protein organization*

The data in table 1 and fig.4 show that increasing cell density caused marked changes in the repartition of detergent-soluble and -insoluble proteins. The proportion of the polymerized (detergent-insoluble) form of actin was reduced by 44.7% whereas the soluble monomeric form was not affected. This reduction in relative rate of synthesis of polymerized actin accounted for the decrease in total actin biosynthesis. The proportion of detergent-insoluble α-actinin was markedly

Table 1

Cytoskeleton synthesis and organization in mouse osteoblasts cultured initially at low cell density (LCD) (4000 cells/cm^2) and high initial cell density (HCD) (8000 cells/cm^2)

	Actin		α-Actinin		Vimentin		α-Tubulins		β-Tubulins	
	LCH	HCD	LCD	HCD	LCD	HCD	LCD	HCD	LCD	HCD
Insoluble + soluble (% total)	1.97	1.77					0.097	0.100	0.226	0.195
% insoluble	4.65	2.57	0.592	0.345	1.28	0.70	0.041	0.017	0.036	0.025
% total	0.80	0.72	0.101	0.097	0.219	0.196	0.007	0.005	0.006	0.007
% soluble	1.41	1.46					0.108	0.133	0.265	0.261
% total	1.17	1.05					0.090	0.095	0.220	0.188

The data represent [^{35}S]methionine-labeled cytoskeletal proteins recovered in detergent (Triton X-100)-soluble (unpolymerized form) and -insoluble (polymerized form) cell lysates and are expressed as the proportion of the total labeled proteins in the insoluble or soluble fractions or relative to the radioactivity in the total (insoluble + soluble) cell lysates

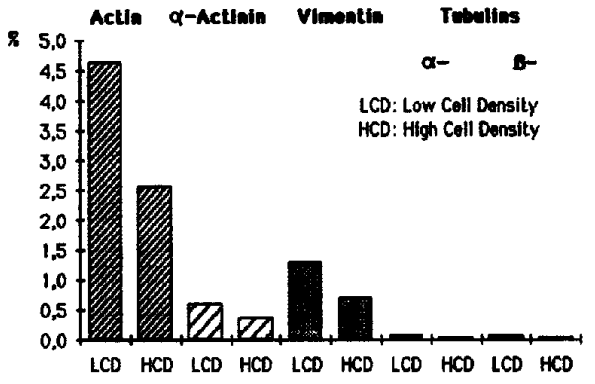

Fig.4. Effect of doubling the initial cell density on the relative rate of synthesis of several cytoskeletal proteins in mouse osteoblastic cells. The cells were initially plated at low density (4000 cells/cm^2) (LCD) or at higher cell density (8000 cells/cm^2) (HCD), cultured for 5 days, labeled for the last 24 h with [^{35}S]methionine and the labeled proteins were identified on radioautograms of 2D gels. The radioactivity corresponding to proteins resolved from insoluble fractions was determined. The data are expressed as the percentage of detergent-insoluble proteins synthesized de novo relative to the total labeled proteins recovered in the insoluble fraction.

decreased by 41.7% (fig.4). Similarly, the proportion of vimentin recovered in the insoluble fraction decreased by 45.3% at high cell density. In addition the biosyntheses of the polymerized forms of α-tubulins and β-tubulins were reduced by 58.5 and 30.6% respectively, with no significant change in the soluble forms.

4. DISCUSSION

We report here for the first time the pattern of synthesis of the most abundant cytoskeletal proteins in mouse endosteal osteoblasts. The biochemical procedures utilized allowed us to identify these proteins by their migration on 2D SDS gels and by immunoreaction with specific antibodies. We have identified the type of microfilaments and microtubules previously observed in osteoblasts and osteocytes by ultrastructural and immunofluorescence methods [7,8]. In addition to actin and tubulins we report the presence of vimentin and α-actinin within osteoblasts. The vimentin-type intermediate filament is expressed in various cell types and has also been found in odontoblasts [18,19], cells which synthesize the dentin matrix. Interestingly, cleavage products of vimentin recognized by the antivimentin antibody were found to occur mainly at high cell density in accordance with findings in other cell types [20].

The pattern of major cytoskeletal elements detected on 2D SDS gels was unchanged with cell density while their rate of synthesis and organization were altered. Cells plated initially at low density exhibited a high relative rate of synthesis of actin, α-actinin and vimentin. Doubling cell density induced a fall in microfilament biosynthesis in total cell lysates. It is known that the synthesis of several intracellular proteins varies with cell density. Actin, vimentin and tubulin syntheses were reported to be regulated by cell-cell contacts and cell shape which affect the expression of genes encoding cytoskeletal proteins [4]. That actin biosynthesis in osteoblastic cells decreased with increased cell number is in accordance with the finding that the expression of actin changes with cell growth [21,22] being decreased when cell spreading is reduced [3,4]. The finding that the synthesis of most (insoluble fraction) α-actinin was reduced at higher cell density suggests that actin-binding proteins synthesis may be altered together with actin. Doubling cell density also affected the relative rate of vimentin synthesis in osteoblastic cells. In other cell types it appears that expression of the vimentin gene may be growth-regulated, being augmented when cells are stimulated to proliferate [23] and being reduced in dense suspension cultures [4]. The occurrence of vimentin cleavage predominantly in dense cell cultures suggests that alteration in the organization of vimentin may regulate the level of vimentin synthesis [20]. These alterations in microfilaments and intermediate filament biosynthesis in osteoblasts related to approach of confluence is consistent with the known effects of the resting state on the synthesis of these proteins [4,24].

Interestingly, we found that the decrease in actin biosynthesis was associated with a marked reduction in the polymerized fraction of actin without change in the depolymerized form. A correlation between the polymerization state and the gene expression of cytoskeletal elements has been described for microtubules [25,26] but it is not known whether this mechanism of regulation also applies to microfilaments. Changes in actin ar-

rangement may trigger actin biosynthesis in osteoblasts as in other cell types [27].

We have found that increasing cell-cell contacts affect the organization and biosynthesis of specific cytoskeletal proteins in osteoblasts. The intriguing hypothesis that hormones that alter osteoblastic cell shape and metabolism also affect cytoskeletal organization as well as synthesis is currently under investigation.

ACKNOWLEDGEMENTS

The authors wish to thank Dr B. Anderton and Mrs F. Landon for providing antibodies and A. Cavalier for typing the manuscript.

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