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Osteopontin involvement in integrin-mediated cell signaling and regulation of expression of alkaline phosphatase during early differentiation of UMR cells

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Abstract To clarify the function of osteopontin in osteoblast differentiation, we have examined the signal transduction pathway in an osteoblastic cell line (UMR106-6) bound to osteopontin, fibronectin, vitronectin and collagen type I surfaces. This was done by investigating the production and autophosphorylation of focal adhesion kinase (FAK) and the expression of alkaline phosphatase (ALP) at the transcription level. Results suggest that osteopontin was not only responsible for the autophosphorylation of FAK but regulated the expression of ALP, which was strongly correlated with FAK activity. These results suggest that osteopontin might act as a trigger in the early differentiation of osteoblasts.

Key words: Osteopontin; Focal adhesion kinase; Osteoblast; Alkaline phosphatase

1. Introduction

Recently, integrin-mediated signal transduction has been widely investigated by biological scientists. Heterodimeric integrin receptors bind to extracellular matrix proteins (ECM) such as laminin (LN), fibronectin (FN), vitronectin (VN), osteopontin (OPN) and collagen type I (CoI). Integrins are widely expressed and involved in important biological functions such as embryonic development, wound repair, homeostasis and the prevention of programmed cell death [1–5]. They are also implicated in tumor-directed angiogenesis, tumor growth and metastasis [6–9]. The binding of integrins to ECM or cells occurs at specialized sites known as focal adhesions. Within a focal adhesion, the cytoplasmic domain of the integrin is linked to the actin cytoskeleton via a complex array of protein-protein interactions. Integrin-ECM binding can activate the signal transduction pathway and regulate gene expression. The binding of integrins to ECM and the activation of inducible pp125 focal adhesion kinase (FAK) are subjects of biological significance which require detailed study.

The major integrins expressed on the surface of human osteoblastic cultured cells are $\alpha_3\beta_1$ [10], $\alpha_4\beta_1$ [11] and $\alpha_v\beta_3$ [12]. On rat osteoblastic cells, the integrin receptors $\alpha_v\beta_3$, $\alpha_v\beta_5$ [13] and $\alpha_5\beta_1$ [14] are expressed, in addition to the above-noted integrin family. FN, CoI, OPN and bone sialoprotein (BSP) are expressed in major bone tissue. Further, the expression of FN, CoI and OPN increases during the initial stage of differentiation in rat calvarial osteoblast cells [15,16]. This increased expression may play an important role in the morphogenesis of the bone and in integrin-mediated cell signaling.

OPN is a secreted highly acidic phosphoprotein which binds to cells via an RGD cell adhesion sequence that recognizes the $\alpha_v\beta_3$ integrin. OPN may regulate the formation and remodeling of mineralized tissue, and may affect gene expression. According to our previous work [17–19], OPN is recognized by osteoblast cells and induces alkaline phosphatase (ALP) activity in vitro. This work indicates that OPN may be involved in the initiation of bone remodeling.

The main purpose of the present study was to determine the effect of OPN on integrin-mediated inducible FAK and its autophosphorylation, and to gain an understanding of what triggers the initiation of early-stage differentiation in the UMR osteoblastic cell line. This was done by observing the gene expression of osteoblast-associated ALP.

2. Materials and methods

2.1. Materials

Anti-FAK rabbit polyclonal antibody, which works against proteins with sequences corresponding to amino acids 903–1025 mapping at the carboxy terminus of FAK of mouse origin, and *p*-Tyr (Py20) monoclonal antibody, which works against phosphotyrosine residues in phosphorylated protein, were purchased from Santa Cruz Biotechnology Inc., CA, USA. The protein A-agarose was obtained from Calbiochem Co., MA, USA. TRIzol reagent was obtained from Life Technologies. The RNA LA PCR Kit (AMV) Ver. 1.1 was purchased from Takara La Biochemical Inc., Japan. The fibronectin and collagen type I were from Sigma Co., MO, USA and Wako Pure Chemical Industries Ltd., Japan, respectively. OPN was purified from newborn rat calvaria by the normal procedure with modification in our laboratory [19]. Other reagents and chemicals were commercially available with analytical purities.

2.2. Cell culture

UMR 106-6 cells were donated by the group headed by Prof. Jaro Sodek at the University of Toronto. The cells were cultured in α-MEM containing 167 units/ml penicillin G, 10^{-6} M dexamethasone, 50 μg/ml gentamicin and 0.3 μg/ml amphotericin B. The α-MEM was supplemented with 15% fetal bovine serum and 50 μg/ml of freshly prepared ascorbic acid, and cultured at 37°C with 5% CO_2.

2.3. Cell adhesion

Six-well plates (3.5 cm in diameter) were coated with various extracellular matrix proteins. The concentrations of FN, VN, and OPN

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Abbreviations: SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate buffered saline; BSA, bovine serum albumin; α-MEM, α-minimal essential medium

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in a coating buffer (of 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) were 50 μg, 25 μg and 25 μg/ml respectively. The concentration of CoI in an acidic solution (of 2 N HCl pH 3.0, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) was 500 μg/ml. All the plates were incubated overnight at 4°C. Then they were blocked with a blocking buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ containing 3.5% BSA) and incubated at 37°C for 1 h. The coated plates were washed with PBS twice before use. Then the cells were resuspended, plated on wells at a density of $1–2\times10^6$, and incubated.

2.4. Immunoprecipitation

The culture medium was carefully removed with a pipette and the monolayer cells were washed twice with PBS. After the addition of 0.8 ml RIPA buffer (PBS pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF), the cells were scraped with a policeman. Using a syringe fitted with a 21-gauge needle, the lysate was pipetted and put into an Eppendorf tube. The well was washed once with 0.3 ml RIPA buffer and combined with the first lysate, and then incubated for 30 min on ice. The lysate was centrifuged at 3000 rpm for 20 min at 4°C and the pellets were discharged. For preclarification, 10 μl (i.e. 1 μg) of protein A conjugated with agarose was added and incubated at 4°C for 1 h. After centrifuging the tubes at 1500 rpm at 4°C for 5 min, the supernatant was transferred to a new tube. 10 μl (approximately 1 μg) of antibody was added and incubated at 4°C for 1 h. 20 μl of protein A-agarose solution was added to each tube and incubated overnight at 4°C with shaking on a racker plate form. After centrifuging at 2500 rpm at 4°C for 5 min, the precipitate was washed four times with 1 ml RIPA buffer. Then 40 μl of SDS-PAGE sample buffer was added to the precipitate and it was heated at 100°C for 3 min. All samples were applied to 7.5% SDS-PAGE gel [20].

2.5. SDS-PAGE, silver staining and Western blot analysis

Samples containing equal protein concentrations were electrophoresed on 7.5% SDS-PAGE gels. After electrophoresis, gels were stained with silver staining reagents or transferred to nitrocellulose. After blocking the nitrocellulose, the FAK and/or phosphorylated FAK were visualized by incubation with the related antibodies, followed by incubation with HRP-labeled second antibodies for staining.

2.6. RNA extraction

4 ml of UMR cells with a density of $1–1.7\times10^6$ cells/ml was resuspended into dishes (10 cm in diameter) coated with FN, OPN, and CoI, respectively. The cells were incubated at 37°C with 5% CO_2. After incubation, all the dishes were washed twice with PBS, pH 7.4. 1 ml of PBS was added to each dish, and all dishes were then placed on ice for 10 min. Then the attached cells were carefully scraped and the dishes were rinsed with 0.2 ml of PBS. After the cells were collected, 1 ml TRIzol reagent was applied into each tube, passing the lysate several times through a pipette. Then the tubes were incubated for 5 min at room temperature. After that, 0.2 ml of chloroform was added and the tubes were shaken vigorously by hand for 12 s. The tubes were left to stand at room temperature for 3–5 min. Then the colorless upper phase was carefully transferred to a new tube. After the addition of 0.5 ml isopropyl alcohol, this tube was incubated at room temperature for another 10 min, then centrifuged at $12\,000\times g$ for 10 min at 4°C. After washing the gel-like RNA precipitate twice with 75% ethanol, the RNA was dissolved with RNase-free water and stored at −20°C until use.

2.7. Reverse transcription and polymerase chain reaction (RT-PCR)

The primers for rat alkaline phosphatase (BLKP ALP) were as follows: P1: 5′ CTT CCC ACC CAT CTG GG 3′; P2: 5′ CTC CCG CCA CGG CGC TC 3′. For β-actin, which was used as an internal standard, the primers were: PF: 5′ CTT CCT TCC TGG GCA TGG AG 3′; PR: 5′ TGG AGG GGC CGG ACT CGT CA 3′. The lengths of the PCR products for ALP and β-actin were 129 and 315 bp respectively.

Reverse transcription was carried out under the following conditions. 20 μl of the reaction mixture contained 4 μl of 25 mM $MgCl_2$, 2 μl of 10×RNA PCR buffer, 2.5 μl of RNase free water, 8 μl of dNTP mixture (2.4 mM each), 20 U of RNasin, 5 U of reverse transcriptase, 20 pmol of oligo-dT adaptor and 1 μg of template RNA. The reaction mixtures were incubated at 55°C for 30 min. Then they were heated at 99°C for 5 min, chilled at 5°C for 5 min, and transferred directly to the PCR reaction.

The PCR reaction mixture contained 6 μl of the RT reaction mixture, 6.75 μl of 25 mM $MgCl_2$, 9 μl of 10×La PCR buffer, 61.5 μl of pure water, 2.5 U of Takara La Taq DNA polymerase, and 25 pmol of each primer. If necessary, 4 μl of dNTP mixture (2.4 mM each) was added. The final volume of this reaction mixture was 100 μl. The running program for amplification was pre-heating at 94°C for 2 min. The cycle program for the ALP was 94°C, 45 s; 50°C, 30 s; 72°C, 2 min, for 40 cycles. For the β-actin, the program was 94°C, 1 min; 60°C, 1 min; 72°C, 1 min, and the number of cycles was 34. The PCR products were analyzed on 2% agarose gel and stained with ethidium bromide.

3. Results

3.1. Production and autophosphorylation of FAK induced by UMR cells attached to ECM

In bone matrix, the main protein is CoI. Non-collagenous protein contains FN, OPN, BSP, osteonectin, osteocalcin, etc. It has been shown that, during the early differentiation of osteoblast cells, OPN is expressed at the transcription level earlier than the other matrix proteins [21]. One of our previous studies has indicated that OPN is recognized and attached more easily by osteoblast cells than is CoI [19]. This suggests that OPN may play an important role in the early differentiation of osteoblasts, possibly because it is an autocrine phosphoprotein with diverse functions [22].

In the present study, we compared the production and autophosphorylation of FAK induced by UMR cells attached to FN, VN, OPN, and CoI surfaces, as well as to non-coated plastic wells (as a control). UMR cells with a density of 2.47×10^6 per well were placed on the ECM and incubated for 60 min at 37°C. Immunoprecipitation with anti-FAK antibodies was then carried out.

Fig. 1A shows the FAK content in UMR cells which was induced by several matrix proteins. The uncoated well acted as a negative control. The data show that CoI induced a high level of FAK generation, which was 5.55 times higher than for BSA-coated wells (not shown), 3.63 times higher than for OPN, and 4.0 times higher than for FN. In sum, the ability to induce FAK was as follows: CoI > VN > OPN > FN.

FAK autophosphorylation, which is activated by UMR cell-matrix protein interaction, was also investigated, by detecting phosphotyrosine residues with the *p*-Tyr antibody. These results are shown in Fig. 1B. OPN showed a significant ability to induce FAK autophosphorylation, and FN showed a similarly high level of inducibility. CoI and VN had little effect on FAK phosphorylation.

The proportion of autophosphorylation to content of FAK

Table 1
Induction of FAK specific activity and ALP expression after UMR cells attached to osteopontin and collagen type I

	Non-adhesive cells	Osteopontin ($r=0.59$)			Collagen type I ($r=-0.07$)		
		30 min	60 min	90 min	30 min	60 min	90 min
FAK specific activity	0	2.xs02	1.18	1.61	2.71	0.22	0.53
ALP expression	0.54	2.34	1.20	0.47	1.33	1.51	1.52

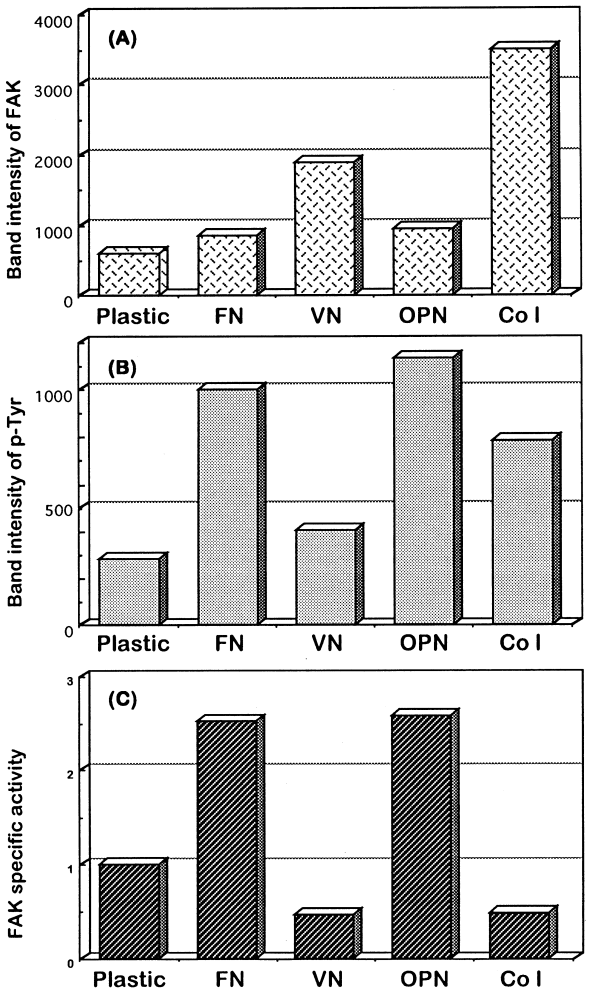

Fig. 1. Content and autophosphorylation of FAK in UMR cells attached to ECM proteins. A: FAK content (FAK); B: FAK autophosphorylation in UMR cells cultured on non-coated plastic wells (control), fibronectin (FN), vitronectin (VN), osteopontin (OPN) and collagen type I (CoI) for 1 h after subculture. C: Relative FAK specific activity (expressed as the ratio of FAK autophosphorylation to FAK content) in UMR cells cultured on ECM substrates.

was calculated, as shown in Fig. 1C. This value was termed 'FAK specific activity'. Although there was a high content of FAK in the UMR cells cultured on CoI and VN surfaces, the calculated ratios indicate that OPN and FN had a significantly greater effect on the phosphorylation of FAK on tyrosine than did VN and CoI. This may mean that OPN and FN play an important role in FAK-mediated signal transduction via cell surface integrin-ECM interaction.

3.2. Kinetics of FAK in UMR cells plated on OPN and CoI surfaces

In order to find out whether matrix proteins play a role in generating FAK and which extracellular protein is responsible for FAK phosphorylation in the early stages of cell attachment to ECM, it is necessary for time courses to be carried out. In the present study, UMR cells were placed on coated wells and incubated for 30, 60 and 90 min. The data obtained are shown in Fig. 2A. (The data for 0 min were from cells kept in suspension.) These data suggest that FAK production induced by OPN gradually increased and then saturated. On the other hand, CoI induced FAK generation up to a maximum at 60 min after incubation.

The time courses for FAK autophosphorylation are shown in Fig. 2B. OPN induced FAK autophosphorylation with a gradual increase. In contrast, the autophosphorylation induced by CoI reached a maximum at 30 min.

The time courses for the specific activity of FAK are given in Fig. 2C. At 30 min, the specific activities for both OPN and CoI were similar; however, at 60 and 90 min there was a significant difference between the FAK activity induced by the respective proteins. CoI has been reported to express much later than OPN [21], which indicates that only OPN is responsible for FAK specific activity during the early stages of UMR differentiation. This proves that FAK induction was transient.

3.3. ALP gene expression in UMR cells attached to OPN, CoI and FN

ALP is known to act as an early differentiation marker in osteoblast differentiation. In this study, RT-PCR was employed in order to detect ALP mRNA expression. β-Actin was used as an internal standard. After separation on agarose gel, the bands which had been detected were scanned, and the

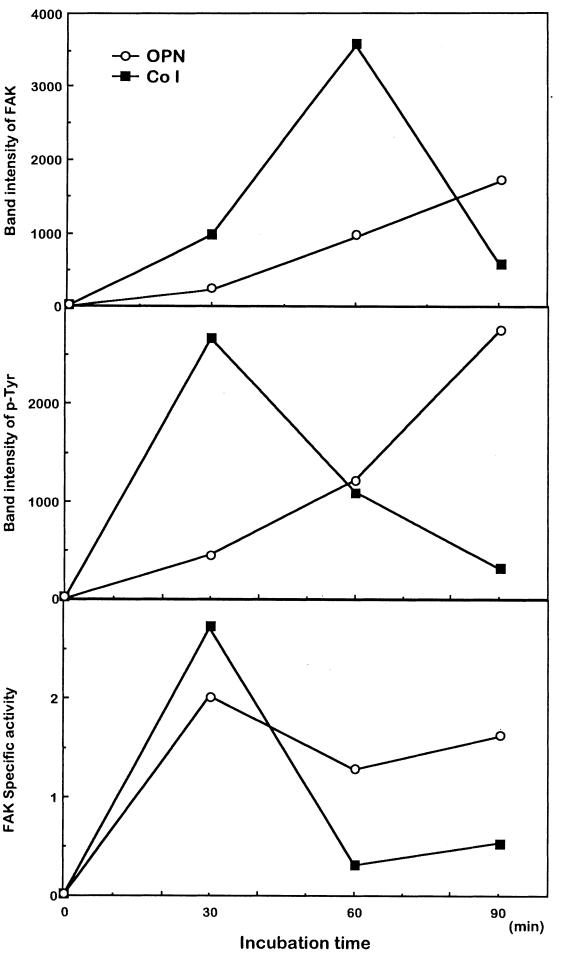

Fig. 2. Kinetics of production and phosphorylation of FAK in UMR cells cultured on OPN and CoI surfaces. A: Time courses for the production of FAK. B: Time courses for the autophosphorylation of FAK. C: Specific activity of FAK after the UMR cells were plated on OPN and CoI surfaces for 30, 60 and 90 min. The data at 0 min were from cells kept in suspension.

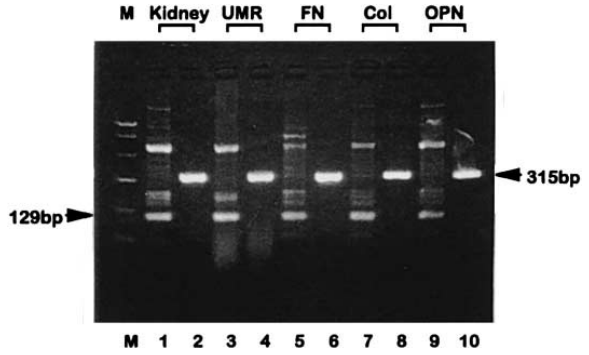

Fig. 3. ALP gene expression in UMR cells attached to FN, CoI and OPN. M: PCR markers, from top to bottom, are 1000, 750, 500, 300, 150, 50. Lanes 1, 3, 5, 7, 9: PCR products of ALP. Lanes 2, 4, 6, 8, 10: PCR products of β-actin. Kidney: cultured kidney cells, as a positive control. The ratio of RT-PCR products of ALP to those of β-actin (internal standard) indicate the semi-quantitative values of ALP gene expression at the transcription level. All data were obtained as the mean value from at least three individual experiments.

density of each band was measured using NIH image software (Fig. 3). Then the ratio of ALP to β-actin was calculated. The time courses for ALP expression are shown in Table 1. When UMR cells were kept in suspension, a low level of ALP expression was detected. During the 90 min period after cells were plated to the substrates, ALP expression induced by FN and CoI stayed at a relatively low level. There was no significant change during this period. In contrast, ALP mRNA expression induced by OPN reached a maximum at 30 min. Further, at 30 min, the production of ALP mRNA induced by OPN was 2.06 and 1.68 times greater than that of FN and CoI respectively. This indicates that OPN was responsible for the early stage induction of ALP expression, while FN and CoI functioned to maintain the ALP level within the cell. Further statistical analysis reveals that ALP mRNA expression correlated to FAK specific activity in the case of OPN induction (r=0.59), but not in that of CoI induction (r=−0.07). In sum, during the early stages of UMR cell differentiation, OPN expression occurred earlier than CoI expression. This indicates that ALP expression may be regulated mainly by OPN.

4. Discussion

OPN is known as an adhesive protein which appears in bone matrix. Previously, we compared the expression of several matrix proteins (CoI, FN, OPN, BSP, osteonectin and osteocalcin) in rat bone marrow osteoblastic primary cells (RBMO), using the immunofluorescence technique [18]. We found that among these proteins, OPN, BSP and CoI may play an important role in the early differentiation of RBMO. Moreover, cell adhesion and ALP assays of RBMO cells have suggested that pre-osteoblasts differentiate into osteoblasts by recognizing OPN [19]. However, until now these have been only hypotheses, requiring detailed experiments such as those carried out in this study to investigate the pathway of signal transduction via integrin-ECM binding in osteoblasts.

It is well known that multiple integrins have multiple ligands. FN has several integrin receptors, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_1$. VN has $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_3$. Collagen has $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$, as well as $\alpha_v\beta_3$. In contrast to these, OPN's only receptor is $\alpha_v\beta_3$. Evidence is emerging that individual integrins bound to ECM on the cell surface mediate distinct functions, trigger distinct signal pathways, and also lead to different cell behaviors [23].

FAK may be a component of the signal transduction pathway and may be responsible for the transmission of signals from cell surface integrins into the cell [23,24]. When BALB/c 3T3 cells and NIH3T3 cells have been plated onto plastic and/or FN surfaces, FAK in both unphosphorylated and phosphorylated form has been detected in these cells, but not in cells kept in suspension [25]. This indicates that FAK can be induced by FN. The same results have been observed from spreading cells onto VN surfaces [26]. This indicates that FAK can be induced by plating cells onto ECM surfaces. FAK generation and autophosphorylation/dephosphorylation are different events within cells. Here, in the case of spreading UMR cells onto ECM surfaces, our results showed that both the production and phosphorylation of FAK were induced, but the degree of ability to induce varied. In order to describe the efficiency of integrin-mediated signal transduction, we examined the specific activity of FAK as expressed by the proportion of phosphorylation to content. FAK may be autophosphorylated on tyrosine residues by various signal molecules such as c-Src, PKC and integrin β subunits [27,28], which generally indicated that FAK has been activated. Tyrosine phosphorylation of FAK may regulate FAK activity-mediated signal transduction. Recently, P.D. Tahiliani has found that the integrin β_3 cytoplasmic domain with a highly conserved NPXY motif was responsible for triggering FAK phosphorylation [29].

FAK, presenting only in cellular focal adhesion, might be responsible for phosphorylating the components of focal adhesion – for example, tenascin, paxillin or talin – and regulating the interaction of integrin with the cytoskeleton and/or the extracellular matrix. In the present study, after plating UMR cells to ECM for 30 min, active FAK was strongly induced by both FN and OPN, but not by VN or CoI. Also, FN has recently been proven to be an important participant in osteoblast differentiation and bone formation from day 8 to day 23 of this process [16].

D.T. Denhardt and his colleagues have indicated that OPN can alter gene expression at the transcription level. This implies the existence of a signal transduction cascade leading from a cell surface receptor to a nuclear protein involved in regulating mRNA transcription. For instance, OPN could reduce the expression of iNOS mRNA in PTE cells. It has been found that this kind of reduction can be inhibited by the OPN antibody and the RGDS peptide [29]. In the present study, ALP expression at the transcription level was a biological marker indicating the early differentiation of UMR cells in vitro. During the first 30 min, ALP expression induced by OPN was much higher than that induced by CoI and FN. At 30 min, the specific activity of FAK induced by OPN and CoI was at about the same level. However, CoI did not increase the expression of ALP. During 90 min of incubation, ALP mRNA expression only correlated to FAK activity induced by OPN. The explanation for this is that the response of tyrosine phosphorylation of FAK to a distinct signaling pathway implies the possibility of specific phosphorylation at different tyrosine residues, which may in turn lead to different effector functions [30]. The results of the present study

suggest that OPN may trigger ALP gene expression via integrin-mediated FAK activation.

The present results not only give strong support to our previous work, indicating that OPN is responsible for early differentiation in osteoblasts; they also indicate a difference between the roles of CoI and OPN in osteoblast differentiation. That is, both OPN and CoI are necessary in osteoblast differentiation, but the former has the role of a trigger and the latter has the role of spontaneous induction. Further detailed study of these roles is necessary and is in progress.

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