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Fibronectin Enhances In Vitro Vascular Calcification by Promoting Osteoblastic Differentiation of Vascular Smooth Muscle Cells Via ERK Pathway

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Abstract The process of vascular calcification presents several features similar to osteogenesis in which fibronectin (FN) acts as a regulator of osteoblastic differentiation and the ERK signal pathway is involved. In order to find whether FN promotes the osteoblastic differentiation of vascular smooth muscle cells (VSMCs) through the ERK signal pathway, we investigated the effect of FN on the calcification of VSMCs by using an in vitro cell model. VSMCs cultured in plates with FN (0–20 μg/cm^2) coating were induced to calcify by 10 mM sodium β-glycerophosphate (β-GP). FN exacerbated VSMC calcification in a dose- and time-dependent manner, as indicated by the number of calcifying nodules per slide and the amount of calcium in the deposition. Data from RT-PCR and immunoblotting assay revealed that FN also enhanced the expression of several phenotypic markers of osteoblasts, including alkaline phosphatase (ALP) activity, osteocalcin (OC), and Osf2/Cbfa1, a key transcription factor in osteoblastic differentiation. Furthermore, a specific inhibitor for ERK, PD98059 (10 μM), significantly suppressed the effect of FN on calcification and phenotypic marker expression. These findings seem to suggest that FN enhanced vascular calcification by promoting the osteoblastic differentiation of VSMCs via ERK signal pathway. J. Cell. Biochem. 99: 1343–1352, 2006.

Key words: fibronectin; vascular smooth muscle cell; calcification; differentiation; ERK pathway

Vascular calcification is a common pathological feature of advanced atherosclerotic lesions [Blumenthal et al., 1944], which is associated with clinical complications such as myocardial infarction, impaired vascular tone, dissection in angioplasty, and poor surgical outcome [Umeda et al., 2003]. Previously, vascular calcification was considered as a passive chemical process involving spontaneous deposition of calcium phosphate crystals in necrotic tissue. More recently, however, the involvement of cellular activities has been recognized. Several markers for osteoblastic lineage were detected in atherosclerotic plaques, including collagen I and the non-collagenous matrix proteins, that is, osteocalcin (OC), osteopontin (OPN), matrix GLA protein (MGP), and osteoglycin [Hirota et al., 1993; Ikeda et al., 1993; Rekhter et al., 1993; Shanahan et al., 1994, 1997]. A lot of factors, such as bioactive peptides, oxidized lipids, cytokines, and leptin, were also involved in the regulation of vascular calcification [Parhami et al., 1997; Hsu et al., 2000; Parhami et al., 2001a; Shioi et al., 2002]. In addition, matrix vesicles were characterized as the initiation sites for mineralization [Tanimura et al., 1983].

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Vascular smooth muscle cells (VSMCs) have been found contributing significantly to the regulation of vascular calcification [Giachelli, 2001].

The osteoblast-like transformation of VSMCs and expression of genes and proteins of the transformed cells are important in vascular calcification [Watson et al., 1994; Topouzis and Majesky, 1996; Gadson et al., 1997]. Physiologically, the proteins expressed by normal VSMCs, for example, MGP [Luo et al., 1997] and OPN [Giachelli and Steitz, 2000] inhibit calcification actively. However, in response to pro-calcification factors, the process is complicated by the osteoblast-like transformation of VSMCs [Steitz et al., 2001], and the transformed VSMCs express ossification-associated genes and proteins which finally lead to formation of mineralized nodules [Mohler et al., 2001]. Besides, these transformed cells respond to factors associated with vascular calcification, resulting in increased mineralization in vitro [Parhami et al., 2001b].

In the development of the atherosclerotic lesion, the role of fibronectin (FN) has rarely been addressed in the re-differentiation of VSMCs into osteoblast-like cells when the relevant stimuli exist. FN presents in the early stages of osteogenesis. The localization of FN in the periosteum of rat calvaria and in the osteoid surrounding implants indicates that FN is synthesized and deposited in the areas of bone tissue where recruitment and commitment of osteoblast precursors occur [Weiss and Reddi, 1980; Moursi et al., 1996]. FN is involved in cell adhesion, proliferation, migration, and differentiation [Katow and Hayashi, 1985]. In arteries, the destroyed integrity of the basement membrane of the injured vessel wall may cause the exposure of VSMCs to an FN-riched extracellular matrix, in which FN is originated from infiltrating plasma and later from the cells within the vascular wall [Morla et al., 2000]. And FN was reported to promote the transition of isolated rat aortic smooth muscle cells from a contractile to a synthetic phenotype [Hedin et al., 1988]. Based on the effect of FN on the matrix composition, calcified nodule formation and alkaline phosphatase (ALP) of calcifying bovine aortic cells, Watson et al. [1998] hypothesized that the development of arterial calcification might be regulated by FN via integrin-based signal pathway.

In the present work, we have investigated the effect of human plasma FN on the calcification of VSMCs by using an in vitro cell model. Calcium deposition, the expression of osteoblastic phenotypic markers, and the changes caused by an inhibitor to ERK have been detected, trying to find whether FN promotes the osteoblastic differentiation of VSMCs through the ERK signal pathway.

MATERIALS AND METHODS

Purification of Human Plasma FN

Fresh human plasma was obtained from Red Cross Blood Bank of Beijing. Plasma (50 ml) was centrifuged (30 min, 4,000*g*) and filtered (3 μm Sartorius filter). FN was extracted from the plasma by gelatin-agarose affinity chromatography and further purified by heparin-agarose affinity chromatography as described by Poulouin et al. [1999]. The concentration of FN was determined with Lowry method [Lowry et al., 1951].

Fibronectin Coating of the Culture Plates

The FN solution (1.0 mg/ml) was applied to 60 mm culture plates and incubated at 37°C overnight to yield surface densities of 5∼30 μg FN/cm^2. The FN-coated plates were then blocked in 1% bovine serum albumin (BSA) for 2 h. The dishes were rinsed twice with phosphate-buffered saline (PBS) prior to the following experiments [Chen et al., 1994].

Cell Culture and In Vitro Calcification

VSMCs were isolated from rat aortic media and identified as previously described [Smith and Brock, 1983]. The culture media was DMEM (high glucose, 4.5 g/L, GIBCO BRL) containing 15% fetal bovine serum (FBS, Hyclone) and 1 mM sodium pyruvate. The cells between passages three and seven were used for all experiments. The cells were plated at a density of 5×10^5 cells in 60 mm tissue culture dishes either coated with purified FN or uncoated. VSMCs calcification was induced with the procedure described by Shioi et al. [1995]. Briefly, after confluence, the cells were inoculated in DMEM calcifying medium containing 15% FBS and 10 mM sodium pyruvate in the presence of 10 mM sodium β-glycerophosphate (β-GP), 1.0×10^{-7} M insulin, and 50 μg/ml ascorbic acid (approximately 284 μM) for 12 days. The media were replaced with fresh one every 2 or 3 days. The transformation to calcifying cells was characterized by

the appearance of multilayer nodules undergoing calcification detected by Alizarin Red S staining. The cells at this stage were used as calcifying VSMCs for the following experiments.

Quantification of Calcium Deposition

Quantification of calcium deposition in extracellular matrix was performed according to Wada et al. [1999]. Briefly, the culture of calcifying VSMCs was decalcified with 0.60 M HCl for 24 h. The calcium content of HCl supernatant was determined by atomic absorption spectroscopy. After decalcification, the cells were washed three times with PBS and solubilized with 0.10 M NaOH/0.10% SDS, and the total protein content was determined with Lowry method [Lowry et al., 1951]. The calcium content of the cell layer was normalized to protein content.

Characterization of Calcifying Nodules by Alizarin Red S Staining

Cells grown in 60 mm plates were washed with PBS three times, and then fixed in 10% formaldehyde in PBS for 45 min at 4°C. The cells were washed with distilled water and then exposed to 2.0% aqueous Alizarin Red S solution (Sigma) for 5 min.

Alkaline Phosphatase Activity Assay

Calcifying VSMCs were washed three times with PBS, and the cellular proteins were solubilized with 1.0% Triton X-100 in 0.90% NaCl and centrifuged. ALP activity in supernatants was assayed with the ALP assay kit (Sigma) as described previously [Parhami et al., 1997]. One unit was defined as the activity producing 1.0 nmol of ρ-nitrophenol per 30 min. ALP activity was normalized to total protein content of the cell layer.

Semiquantitative RT-PCR Analysis

Total cellular RNA was extracted using Trizol reagent according to the manufacturer's instruction (Invitrogen). RT-PCR was performed with the SuperScript one-Step RT-PCR system and gene-specific primers according to the condition recommended by the manufacturer (Invitrogen). Reaction mixtures, containing total RNA (500 ng of each), 0.20 mM dNTPs, 0.80 μM of each primer, 2.0 units of enzyme mixture containing SuperScript II RT, Platinum Taq DNA polymerase, and buffer with 1.2 mM $MgSO_4$, were kept at 50°C for 30 min, then at 94°C for 2 min. PCR was performed as follows: the PCR profile was 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The primers for RT-PCR were designed based on rat sequences in Genebank. These sequences are listed in the supplementary table.

Immunoblotting

Cells were lysed with 200 μl of ice-cold buffer containing 10 mM Tris, pH 7.9, 10 mM KCl, 2.0 mM $MgCl_2$, 0.10 mM EDTA, 0.70% Nonidet P-40 on ice for 10 min and centrifuged at 500*g* for 5 min. The cytosolic extracts were harvested to measure ERK activation and the expression of SM-α-actin and SM22α. The nuclear pellets were further lysed with buffer containing 40 mM Tris, pH 7.9, 350 mM NaCl, 2.0 mM $MgCl_2$, 1.0 mM EDTA, 0.20 mM EGTA, 20% glycerol, 1% Nonidet P-40, 1.0 mM phenylmethylsulfonyl fluoride, 2.0 μM dithiothreitol, 2 μg/ml leupeptin, and 1.0 μg/ml aprotinin on ice for 20 min, and then harvested to determine Cbfa-1 activation by centrifugation at 12,000*g*, 4°C, for 10 min. Protein concentrations in cytosolic and nuclear extracts were determined by Lowry method. Aliquots (80 μg in total protein) were subjects to SDS–PAGE and then transferred to nitrocellulose membrane by electrophoresis. The membrane was blocked with 10% defatted milk powder solution in TBS (Tris balanced saline, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 2 h, then blotted with appropriate antibodies (Santa Cruz Biotechnology) in 5% BSA/TTBS (0.02% Tween 20 in TBS) at 4°C overnight and incubated with an IgG in 5% BSA/TTBS for 1 h. The bound antibody was detected in situ using the protocols provided by the manufacturer (Vigorous Biotechnology, Beijing). The optical densities of bands were quantified by a Scion Image software.

Signaling Pathway in the FN Promoted VSMCs Osteoblastic Differentiation

To find out the downstream intracellular signaling pathways, we explored the relationship between ERK phosphorylation of VSMCs and the FN treatment investigated. VSMCs were treated with 15 μg/cm^2 FN for 12, 24, or 36 h and the cell lysates were subjected to immunoblotting with anti-phosphorylated ERK1/2 antibody. To further confirm the relationship between FN and ERK, we used PD98059, which inhibits MEK, an upstream

molecule of the ERK phosphorylation cascade, to see whether the cell differentiation is inhibited. VSMCs were cotreated with FN and PD98059. Nodule formation, Ca deposition, and ALP activity were assessed after 12 days. Cbfa-1 activation and OC RNA level were assessed after 2 days.

Statistical Analysis

Data are the mean ± SD of independent experiments (n = 3). Means were compared by one-way ANOVA, with comparison of different groups by Fisher's protected least significant different test. A value of $P < 0.05$ is considered significant.

RESULTS

FN Enhanced VSMC Calcification

VSMC calcification was induced by β-GP, and was enhanced by FN. After 10 days, calcifying nodules appeared in FN-treated VSMCs (Fig. 1B), but not in the control (Fig. 1A). Two more days were needed for the observation of calcifying nodules in VSMCs cultured in normal plates. Both the number of the calcifying nodules per slide (Fig. 1C, after 12 days) and the amount of calcium deposition in extracellular matrix (Fig. 2A, FN 15 μg/cm^2; Fig. 2B, after 12 days) were significantly increased in FN-treated VSMCs.

FN Increased ALP Activity and the Expression of Osteoblastic Differentiation Markers in CVCs

Alkaline phosphatase was well established to be one of the phenotypic markers of osteoblastic differentiation and a critical enzyme in calcification. The effect of FN on ALP activity in CVCs was found in parallel with calcium deposition. Both the dose dependence (Fig. 2D, after 12 days) of ALP activity on the FN content and the time dependence (Fig. 2C, FN 15 μg/cm^2) were observed. Furthermore, the expression of the ALP mRNA was upregulated in CVCs after culture in FN-coated plates (Fig. 3A, 15 μg FN/cm^2) for 48 h. Similar results were also observed for the mRNA expression of other phenotypic markers, such as OC and Cbfa-1 (Fig. 3). In addition, data from the immunoblotting experiments revealed an increase in the level of Cbfa-1 and a decrease in the SMC contractile phenotype markers, SM-α-actin and SM22α, with increasing FN concentration after 5 days (Fig. 4). Here β-actin was a loading control.

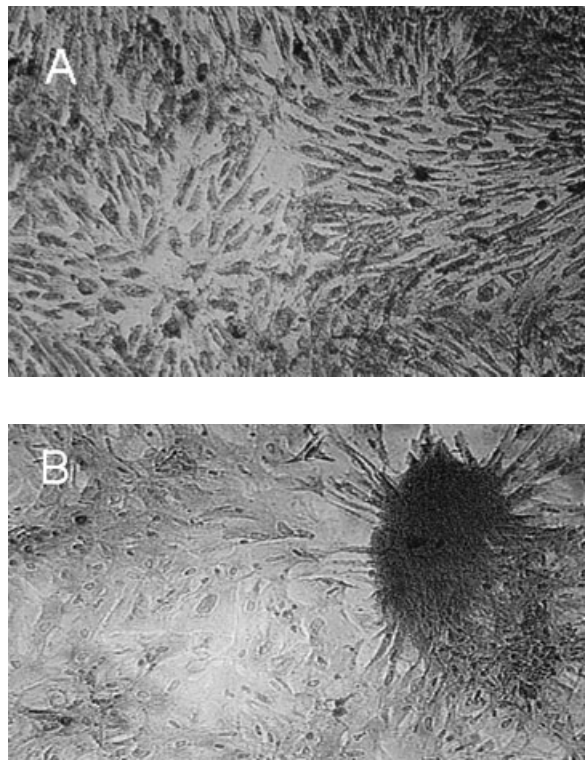

Fig. 1. Effect of FN on calcifying nodule formation by VSMCs. VSMCs were treated with coating FN (**B**, 15 μg/cm^2) or without FN (**A**, control) for 10 days (phase contrast images, original magnification 40×). Typical CVCs were evident in both the groups. Alizarin Red S staining demonstrated mineralized nodules in the FN-treated CVCs, but no nodules were observed in the control. **C**: Cells seeded in glass slides were cultured in calcifying medium and medium containing FN (15 μg/cm^2) after 12 days. Calcifying nodules were counted after stained by Alizarin Red S method (mean ± SD, n = 3). *$P < 0.05$ compared with calcifying control group.

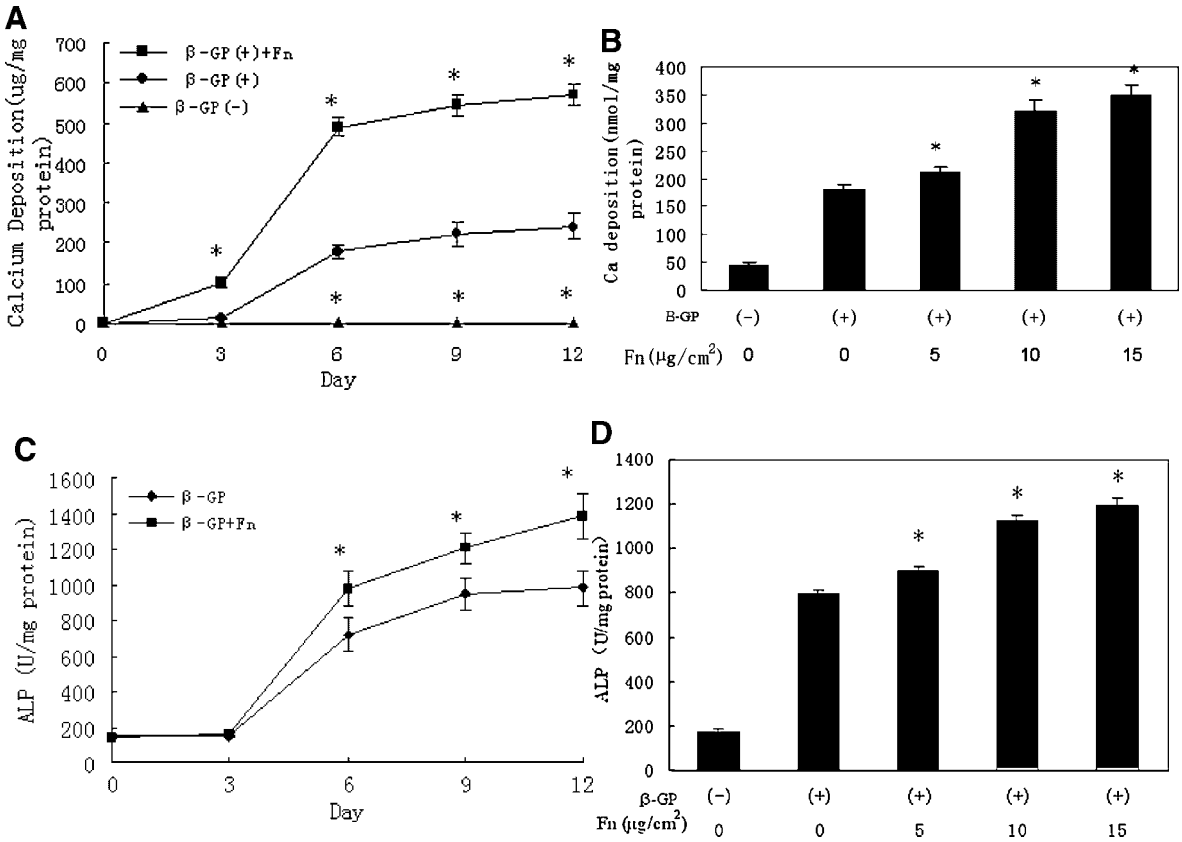

Fig. 2. Effects of FN on VSMC Ca deposition and ALP activity. **A**: Time-dependent effect of FN. Cells were cultured in calcification medium for the indicated time periods in the presence of 15 μg FN/cm^2 as described in Methods. Calcium contents of the cell layers were measured by atomic absorption spectroscopy method, normalized to cellular protein content, and are presented as mean ± SD (n = 3). Differences compared with calcified controls at each time point were statistically significant (*$P < 0.05$, Fisher's protected least significant difference test[PLSD]). **B**: Dose-dependent effect of FN. Calcium contents of the cell layers were assessed as described above and are presented as mean ± SD (n = 3). Difference compared with calcified control were statistically significant (*$P < 0.05$, Fisher's PLSD). **C**: Time-dependent effects of FN. Cells were cultured in calcification medium for the indicated time periods in the presence of 15 μg/cm^2 as described in Methods. ALP activity were measured, normalized to cellular protein contents, and are presented as mean ± SD (n = 3). Differences compared with calcified controls at each time point were statistically significant (*$P < 0.05$, Fisher's PLSD). **D**: Dose-dependent effect of FN. ALP activity were measured, normalized to cellular protein contents, and are presented as mean ± SD (n = 3). Differences compared with calcified controls at each time point were statistically significant (*$P < 0.05$, Fisher's PLSD).

ERK Pathway Was Involved in FN-Promoted VSMCs Osteoblastic Differentiation

Immunoblotting analyses showed that FN activated ERK1/2 (Fig. 5A). And the ERK phosphorylation was dependent on the FN concentration (Fig. 5B). The inhibitor of ERK inhibited both VSMC calcification (Fig. 5C–E) and these osteoblastic differentiation markers (Fig. 5F–G).

DISCUSSION

Vascular calcification is a pathological condition that occurs in many diseases, including atherosclerosis. Atherosclerotic calcification resembles osteogenesis [Bostrom, 2001]. Several studies aiming to verify this hypothesis and exploring the regulating mechanism have been reported [Gadeau et al., 1993; Luo et al., 1997]. Accumulated evidence indicates that the vascular calcification is associated with the osteoblastic differentiation of VSMCs. And the osteoblasic differentiation may be induced via different signaling pathway. The role of extracellular matrix in this aspect is increasingly recognized. As an important extracellular matrix protein, FN has been known to associate with the osteoblast differentiation in bone formation [Glimcher, 1985; Winnard et al., 1995]. Until recently, however,

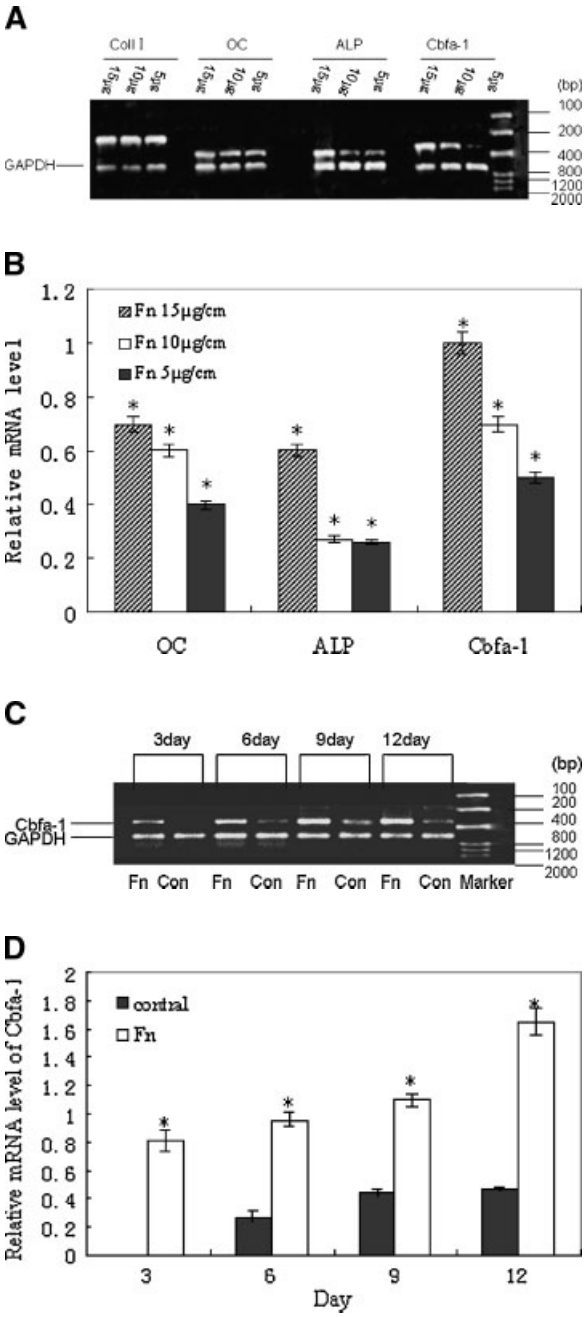

Fig. 3. Effect of FN on CVC differentiation markers. Semiquantitative RT-PCR was performed using specific primers for Cbfa-1 (375 bp), ALP (418 bp), OC (416 bp). **A**: Total RNA was isolated from cells after treatment with 5 μg, 10 μg, and 15 μg FN after 2 days. GAPDH (646 bp) expression was used as an internal control. **B**: Relative expression of OC, ALP, and Cbfa-1, normalized to GAPDH. **C**: RT-PCR of Cbfa-1 levels from cells treated with fibronectin (15 μg/cm^2) at 3 days, 6 days, 9 days, and 12 days. GAPDH expression was used as an internal control. **D**: Relative expression of Cbfa-1, normalized to GAPDH. Data represent results typical of those from three separate experiments.

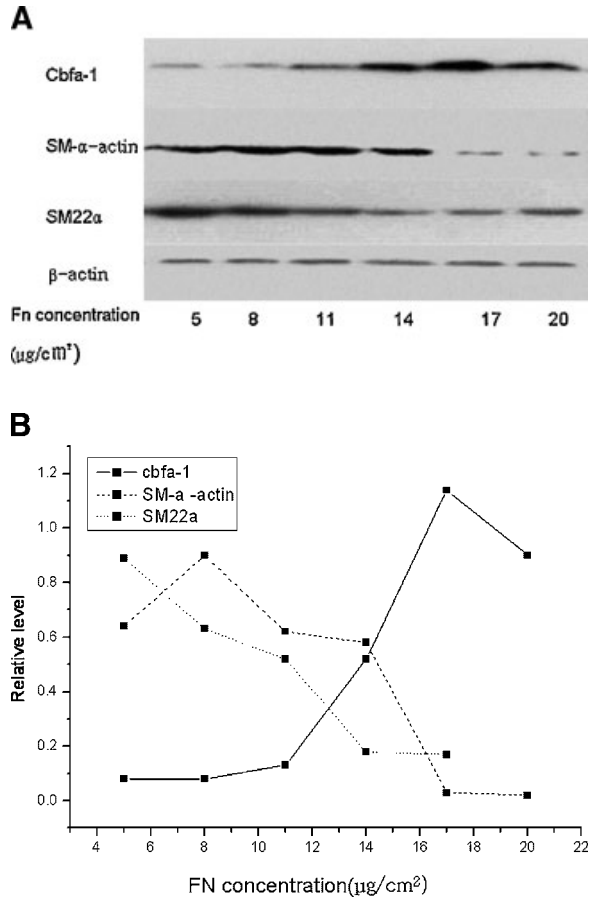

Fig. 4. Effect of FN on CVC differentiation markers. Immunoblotting was performed using specific antibodies for Cbfa-1, SM-α-actin, and SM22α after 5 days. **A**: CVCs were treated with FN at different concentration and the cells lysates were subjected to immunoblotting. **B**: Relative expression of Cbfa-1, SM-α-actin, and SM22α normalized to β-actin.

little was known about the mechanism of FN-regulating vascular calcification. Since the calcified vascular cells with osteoblast-like cell phenotype traits are the cellular basis of vascular calcification [Bostrom, 2001], FN in the blood stream may contribute to the calcification by promoting the osteoblastic differentiation of vascular cells. In this process, the signaling pathway may be different than those induced by cytokines and cellular factors. FN may mediate the osteoblast differentiation and subsequent calcification at least partially by extracellular matrix signaling pathway. To verify the hypothesis, we have studied the effect of FN on vascular calcification and osteoblast differentiation with cultured rat VSMCs treated with β-GP as an in vitro model.

Our data indicate that FN exposure enhanced in vitro vascular calcification by promoting

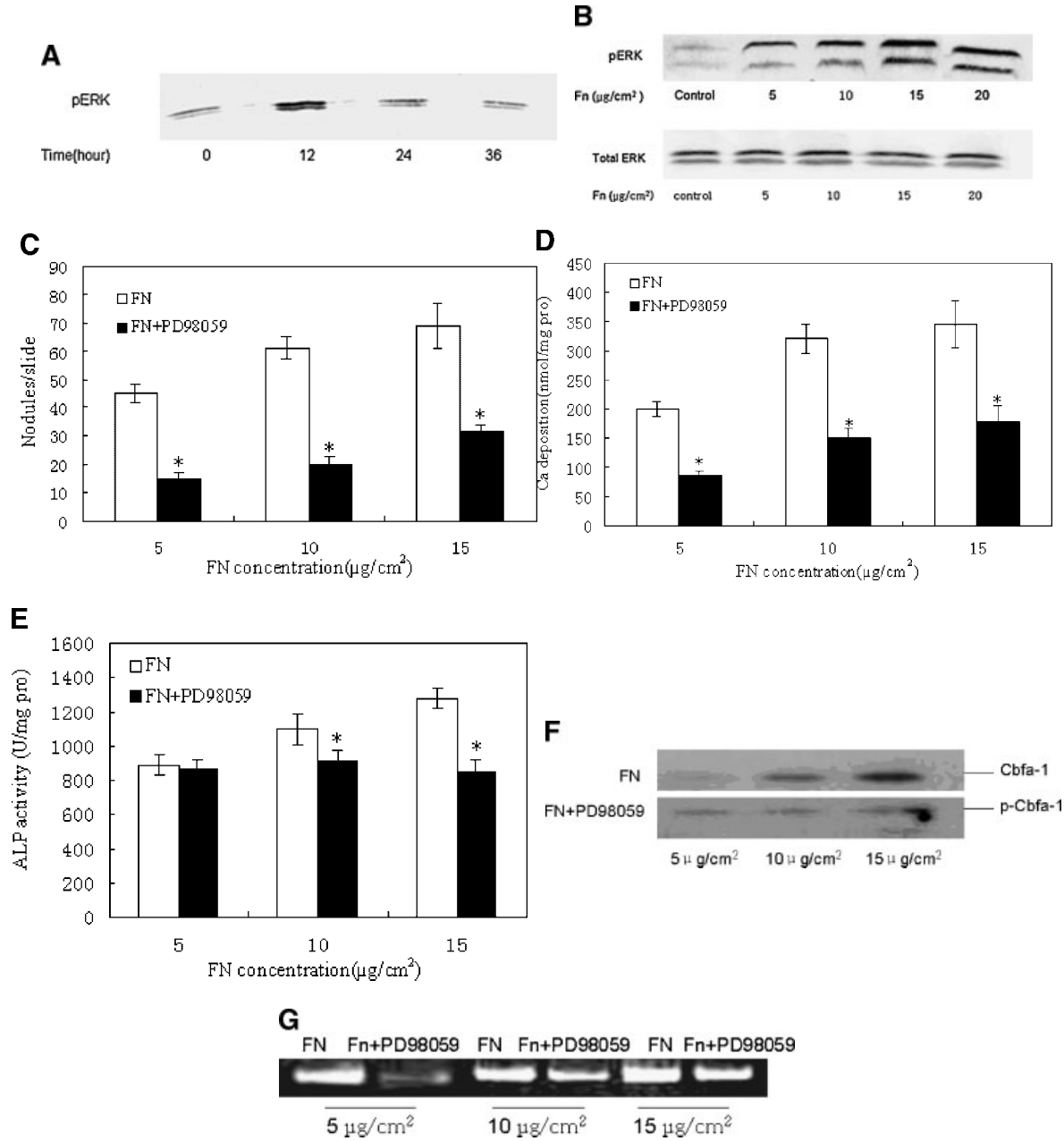

Fig. 5. Effect of FN on ERK phosphorylation. **A**: Western analyses of phosphorylated ERK in CVCs cultures treated with FN (15 μg/cm^2) for the indicated time (n = 3). **B**: Western analyses of phosphorylated ERK and total ERK in CVC cultures treated with different FN concentration for 48 h. **C**: Addition of PD98059 suppressed FN-promoted calcified nodule formation as determined by Aliza Red S method (mean ± SD, n = 3). *$P < 0.05$ compared with calcifying control group after 12 days. **D**: Ca deposition of CVC cultures treated with FN, PD98059 (10 μM) as indicated after 12 days (n = 3). Differences compared with controls at each concentration point were statistically significant (*$P < 0.05$, Fisher's PLSD). **E**: ALP activity of CVC cultures treated with FN, PD98059 (10 μM) as indicated (n = 3). Differences compared with controls at each concentration point were statistically significant (*$P < 0.05$, Fisher's PLSD). **F**: addition of PD98059 (10 μM) blocked FN-induced Cbfa-1 activation. Top lane showed that total Cbfa-1 protein level from CVCs with FN treatment on a Western blot. Bottom lane showed that reciprocal Cbfa-1 activation by tyrosine-phosphorylated Cbfa-1 (p-Cbfa-1). **G**: RT-PCR analyses of OC level treated with FN, PD98059 (10 μmol/L) as indicated.

osteoblastic differentiation of VSMCs. The supporting evidence included (1) the characteristic morphological change indicating VSMCs differentiating into osteoblastic cells; (2) the upregulated osteoblastic differentiation markers, including ALP and osteocalcin; and (3) the upregulated Cbfa-1 expression and reduced contractile phenotype markers SM-α-actin

and SM22α. In addition, the activated ERK1/2 phosphorylation suggests that the FN-promoted differentiation is mediated by ERK pathway. This notion is further supported by the fact that the inhibition of the ERK pathway suppressed FN's effects on nodule formation, ALP activity, Ca deposition, OC expression, and Cbfa-1 activation.

Both calcium deposition in extracellular matrix and ALP activity were increased by FN exposure. These results indicate that FN promoted calcification is associated with increased ALP activity, since ALP can degrade β-GP and raise the local concentration of phosphorus [Gronowicz et al., 1989]. The results are consistent with Watson's finding [1998].

Beside the enhanced expression of OC and ALP, the expression of Cbfa-1, a key regulatory transcription factor in osteoblastic differentiation, was also upregulated. Osf2/Cbfa-1 is important for modulating the expression of osteoblast-specific genes such as OC and ALP [Banerjee et al., 1997; Nakashima et al., 2002]. It plays the role of "master gene," as well as a molecular marker for osteoblastic differentiation. Jono et al. [2000] found that phosphate induced Cbfa-1 upregulation of bovine artery SMCs. Considering these previous findings and the present results, one can deduce that FN promotes cell differentiation by increasing Cbfa-1 expression. Therefore, it is likely that FN promotes osteoblastic differentiation of VSMCs by increasing the expression of the Osf2/Cbfa1 gene and consequently, the level of its downstream transcriptional targets, such as ALP and OC. It is not yet clear whether Cbfa-1 is a solely transcriptional regulator and exerts a direct effect on ALP and OC expression or not.

Moreover, there is a dramatic loss of the contractile markers, SM-α-actin and SM22α, in FN-treated VSMCs. This indicates that under normal conditions, VSMCs express SM-α-actin and SM22α representing the contractile phenotype, while exposure to elevated FN induces VSMCs phenotypic transition. It seems that FN has the capacity to induce SMC differentiation from a contractile to an osteoblastic phenotypic state.

As to the possible signal transduction pathway of FN on VSMC differentiation, the present results showed that FN activated ERK, the specific extracellular signal-regulated kinase of MAPK (mitogen-activated protein kinase) pathway. The MAPK pathway is a major point of convergence for a variety of intracellular signals initiated by ECM–integrin interaction [Xiao et al., 2000]. Previously, some evidence suggested that the differentiation of mesenchymal stem cells to the osteogenic lineage might be regulated by activation via ERK pathway [Jaiswa et al., 2000]. In addition, MAPK pathway may be involved in osteoblast differentiation, of which ERK is essential in the early stages [Lai et al., 2001]. It stimulates the osteoblast-related gene expression by extracellular matrix–integrin receptor interaction, BMP-2, growth factors as well as mechanostressing [Lai et al., 2001; Wang et al., 2002; Xiao et al., 2002; Ziros et al., 2002]. However, how is FN involved in the MAPK pathway as the extracelluar matrix protein? It has been reported that FN activates MAPK pathway through integrin-mediated cell adhesion [Chen et al., 1994]. Integrins are known to activate signaling pathways including the activation of RAS/ERK. Therefore, we hypothesize that FN induces the VSMC differentiation via the MAPK pathway by activating ERK phosphorylation. This is supported by the suppressing effect of ERK-specific inhibitor (PD98059) on nodule formation, ALP activity, Ca deposition, OC expression, and Cbfa-1 activation.

As to the relation between MAPK pathway and Cbfa-1 expression, Xiao et al. [2000] provides the evidence that Cbfa-1 is controlled by MAPK for osteoblastic differentiation. Stimulation of MAPK enhances the expression of OC, whereas the inhibitor PD98059 inhibits the expression. Our data suggest that Cbfa-1 activity could be regulated by MAPK-dependent phosphorylation in CVCs.

In conclusion, our findings and previously reported facts support the hypothesis that FN enhances in vitro calcification by promoting osteoblastic differentiation in rat VSMCs, and this effect might be mediated by the MAPK pathway, in which the increased ERK phosphorylation induces Cbfa-1 expression and the consequent differentiation-associated gene expression, leading to the enhancement of CVCs calcification.

REFERENCES

Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, Lian JB. 1997. Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. J Cell Biochem 66: 1–8.

Blumenthal HT, Lansing AI, Wheeler PA. 1944. Calcification of the media of the human aorta and its relationship to intimal arteriosclerosis, aging and disease. Am J Pathol 20:665–687.

Bostrom K. 2001. Insights into the mechanism of vascular calcification. Am J Cardiol 88(2A):20E–22E.

Chen Q, Kinch MS, Lin TS, Burridge K, Juliano RL. 1994. Integrin-mediated cell adhesion activates mitogen-activated protein kinas. J Biol Chem 269:26602–26605

Gadeau AP, Campan M, Millet D, Candresse T, Desgranges C. Osteopontin overexpression is associated with arterial smooth muscle cell proliferation in vitro. Arterioscler Thromb 1993; 13:120–125.

Gadson PF, Jr., Dalton ML, Patterson E, Svoboda DD, Hutchinson L, Schram D, Rosenquist TH. 1997. Differential response of mesoderm- and neural crest-derived smooth muscle to TGF-beta 1: Regulation of c-myb and alpha 1(I) procollagen genes. Exp Cell Res 230: 169–180.

Giachelli CM. 2001. Ectopic calcification: New concepts in cellular regulation. Z Kardiol 90 (Suppl 3):31–37.

Giachelli CM, Steitz S. 2000. Osteopontin: A versatile regulator of inflammation and biomineralization. Matrix Biol 19:615–622.

Glimcher MJ. 1985. Role of collagen and phosphoproteins in the calcification of bone and other collagenous tissues. In: Rubin R, Weiss GB, Putney JW, editors. Calcium in biological systems. New York, NY: Plenum Press. 607p.

Gronowicz G, Woodiel FN, McCarthy M-B, Raisz LG. 1989. In vitro mineralization of fetal rat parietal bones in defined serum-free medium: Effect of β-glycerol phosphate. J Bone Miner Res 4:313–324.

Hedin U, Bottger BA, Forsberg E, Johansson S, Thyberg J. 1988. Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. J cell Biol 107:307–319.

Hirota S, Imakita M, Kohri K, Ito A, Morii E, Adachi S, Kim HM, Kitamura Y, Yutani C, Nomura S. 1993. Expression of osteopontin messenger RNA by macrophages in atherosclerotic plaques. A possible association with calcification. Am J Pathol 143:1003–1008.

Hsu HH, Tawfik O, Sun F. 2000. Effect of lectins on calcification by vesicles isolated from aortas of cholesterol-fed rabbits. Biochim Biophys Acta 1464:262–272.

Ikeda T, Shirasawa T, Esaki Y, Yoshiki S, Hirokawa K. 1993. Osteopontin mRNA is expressed by smooth muscle-derived foam cells in human atherosclerotic lesions of the aorta. J Clin Invest 92:2814–2820.

Jaiswa RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF. 2000. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. J Biol Chem 275:9645–9652.

Jono S, Mckee MD, Marry CE, Shioi A, Nishizawa Y, Mori K, Morii H, Giachelli CM. 2000. Phosphate regulation of vascular smooth muscle cell calcification. Circ Res 87: E10–E17.

Katow H, Hayashi M. 1985. Role of fibronectin in primary mesenchyme cell migration in the sea urchin. J Cell Biol 101:1487–1491.

Lai CF, Chaudhary SL, Fausto A, Halstesd LR, Ory DS, Avioli LV, Cheng SL. 2001. Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. J Biol Chem 276:14443–14450.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Luo G, Ducy P, Mckee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. 1997. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature 386:78–81.

Mohler ER III, Gannon F, Reynolds C, Zimmerman R, Keana MG, Kaplan FS. 2001. Bone formation and inflammation in cardiac valves. Circulation 103:1522–1528.

Morla AO, Mogford JE. 2000. Control of smooth muscle cell proliferation and phenotype by integrin signaling through focal adhesion kinase. Biochem Biophys Res Commun 272:298–302.

Moursi AM, Damsky CH, Lull J, Zimmerman D, Doty SB, Aota S, Globus RK. 1996. Fibronectin regulates calvarial osteoblast differentiation. J Cell Sci 109:1369–1380.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Parhami F, Morrow AD, Balucan J, Leitinger N, Watson AD, Tintut Y, Berliner JA, Demer LL. 1997. Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. A possible explanation for the paradox of arterial calcification in osteoporotic patients. Arterioscler Thromb Vasc Biol 17:680–687.

Parhami F, Tintut Y, Ballard A, Fogelman AM, Demer LL. 2001a. Leptin enhances the calcification of vascular cells: Artery wall as a target of leptin. Circ Res 88:954–960.

Parhami F, Tintut Y, Patel JK, Mody N, Hemmat A, Demer LL. 2001b. Regulation of vascular calcification in atherosclerosis. Z kardiol 90 (Suppl 3):27–30.

Poulouin L, Gallet O, Rouahi M, Imhoff JM. 1999. Plasma fibronectin: Three steps to purification and stability. Protein Expr Purif 17:146–152.

Rekhter MD, Zhang K, Narayanan AS, Phan S, Schork MA, Gordon D. 1993. Type I collagen gene expression in human atherosclerosis. Localization to specific plaque regions. Am J Pathol 143:1634–1648.

Shanahan CM, Cary NR, Metcalfe JC, Weissberg PL. 1994. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. J Clin Invest 93:2393–2402.

Shanahan CM, Cary NR, Osbourn JK, Weissberg PL. 1997. Identification of osteoglycin as a component of the vascular matrix. Differential expression by vascular smooth muscle cells during neointima formation and in atherosclerotic plaques. Atherioscler Thromb Vasc Biol 17:2437–2447.

Shioi A, Nishizawa Y, Jono S, Koyama H, Hosoi M, Morii H. 1995. Beta-Glycerophosphate accelerates calcification in cultured bovine vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 15:2003–2009.

Shioi A, Katagi M, Okuno Y, Mori K, Jono S, Koyama H, Nishizawa Y. 2002. Induction of bone-type alkaline phosphatase in human vascular smooth muscle cells: Roles of tumor factor-α and oncostain derived from macrophages. Circ Res 91:9–16.

Smith JB, Brock TA. 1983. Analysis of angiotensin-stimulated sodium transport in cultured smooth muscle cells from rat aorta. J Cell Physiol 114:284–290.

Steitz SA, Speer MY, Curinga G, Yang HY, Haynes P, Aebersold R, Schinke T, Karsenty G, Giachelli CM. 2001. Smooth muscle cell phenotype transition associated with calcification: Upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. Circ Res 89:1147–1154.

Tanimura A, McGregor DH, Anderson HC. 1983. Matrix vesicles in atherosclerotic calcification. Proc Soc Exp Biol Med 172:173–177.

Topouzis S, Majesky MW. 1996. Smooth muscle lineage diversity in the chick embryo. Two types of aortic smooth cell differ in growth and receptor-mediated transcriptional responses to transforming growth factor-beta. Dev Biol 178:430–445.

Umeda Y, Mori Y, Takagi H, Iwata H, Matsuno Y, Hirose H. 2003. Surgical outcome of abdominal aortic aneurysm repair in patients undergoing chronic hemodialysis. Heart Vessels 18:7–11.

Wada T, McKee MD, Steitz S, Giachelli CM. 1999. Calcification of vascular smooth muscle cell cultures inhibited by osteopontin. Circ Res 84:166–178.

Wang FS, Wang CJ, Sheen-Chen SM, Kuo YR, Chen RF. Yang KD. 2002. Superoxide mediates shock wave induction of ERK dependent osteogenic transcription factor (RUNX2) and mesenchymal cell differentiation toward osteoprogenitors. J Biol Chem 277:10931–10937.

Watson KE, Bostrom K, Ravindranath R, Lam T, Norton B, Demer LL. 1994. TGF-beta1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify. J Clin Invest 93:2106–2113.

Watson KE, Parhami F, Shin V, Demer LL. 1998. Fibronectin and collagen I matrixes promote calcification of vascular cells *in vitro*, whereas collagen IV matrix is inhibitory. Arterioscler Thromb Vasc Biol 18:1964–1971.

Weiss RE, Reddi AH. 1980. Synthesis and localization of fibronectin during collagenous matrix-mesenchymal cell interaction and differentiation of cartilage and bone in vivo. Proc Natl Acad Sci USA 77:2074–2078.

Winnard RG, Gerstenfeld LC, Toma CD, Franceschi RT. 1995. Fibronectin gene expression, synthesis, and accumulation during in vitro differentiation of chicken osteoblasts. J Bone Miner Res 10:1969–1977.

Xiao G, Jiang D, Thomas P, Benson MD, Guan KL, Karsenty G, Franceschi RT. 2000. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 275:4453–4459.

Xiao G, Jiang D, Gopaladrishnan R, Franceschi RT. 2002. Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. J Biol Chem 277:36181–36187.

Ziros PG, Gil AP, Georgakopoulos T, Habeos I, Kletsas D, Basdra EK. 2002. Papavassiliou AG. The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells. J Biol Chem 277:23934–23941.