Vascular calcification, a significant predictor for cardiovascular events that is present in the majority of patients with clinically significant coronary artery disease, is a highly regulated process resembling embryonic bone formation. We previously isolated, from the bovine aortic media, a subpopulation of cells able to undergo osteoblastic differentiation and mineralization following the molecular time sequence that characterizes differentiation of bone-derived osteoblasts. These phenomena also have been demonstrated in other vascular cells. Atherogenic and inflammatory mediators enhance osteoblastic differentiation of these cells and, therefore, may couple atherosclerosis to calcification.

Insulin-like growth factor I (IGF-I) is expressed in many tissues including bone and acts as a paracrine regulator of osteoblasts. Both IGF and its receptor are also detected in atherosclerotic lesions. IGF-I promotes proliferation, survival, and migration of vascular smooth muscle cells. Patients with low serum IGF-I levels have increased cardiovascular mortality. The effects of IGF-I on vascular calcification are not known.

In this report, we tested the hypothesis that IGF-I regulates vascular calcification in vitro. The effects of IGF-I on proliferation and osteoblastic differentiation of calcifying vascular cells were assessed. Results suggest that IGF-I induces proliferation and inhibits the osteoblastic differentiation and mineralization of the vascular cells via the IGF-I receptor (IGF-IR) activation of extracellular signal-regulated protein kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways.

Materials and Methods

Materials
Recombinant human IGF-I was from Biosource International. PD98059 and LY29402 were from Calbiochem. [Arg3]IGF-I and [Leu24]IGF-I were from GroPep Bioreagents. [3H]Thymidine incorporation was assayed as described. Each bar represents the mean of four wells.

Cell Culture
CVCs, isolated from bovine aortic medial explants, were cultured as described. Treatments were administered in DMEM containing 5% FBS (HyClone Labs) 1 day after plating (at subconfluence), and fresh media with test agents were replenished every 3 or 4 days until assayed.

Alkaline Phosphatase Activity Assay
Alkaline phosphatase (AP) activity from whole cell lysates was assayed and normalized using protein concentration (Bradford assay) as described. Each bar represents the mean of four wells.

45Ca Incorporation Assay
The cell medium was supplemented with 5 mmol/L β-glycerophosphate in addition to the tested agents at the time of first treatment and with every feeding. Twenty four hours before assay, media was supplemented with 45CaCl2 (1.0 μCi/mL). Radiolabeled calcium incorporation was assayed as described. Each bar represents the mean of four wells.

3H-Thymidine Assay
3H-thymidine incorporation was assayed as described. Each bar represents the mean of six wells.
Western Analysis

Whole cell lysates were prepared, and Western analysis performed using standard protocols.

Transfection Assays

Cells (0.5×10^6 cells) were transfected with pFC-MEKK (5 μg; Stratagene) or mock-transfected using Nucleofactor (Amaxa Biosystems). Transfection efficiency was approximately 77%, determined with a plasmid expressing green fluorescent protein (pmaxGFP; Amaxa Biosystems).

Northern Analysis

Total RNA was isolated from confluent CVC culture and analyzed using human IGF-IR cDNA probe (American Type Culture Collection).

Data Analysis

Data are expressed as mean±SD, and means were compared using one-way ANOVA, with comparison of different groups by Fisher protected least significant difference test. A value of P<0.05 was considered significant.

Results

IGF-I Inhibits Osteoblastic Differentiation and Mineralization of CVCs

Treatment with IGF-I for 4 days dose-dependently inhibited AP activity (46±9% at 50 ng/mL; Figure 1A). Matrix mineralization, a marker for osteoblastic differentiation and function, was assessed by ^45Ca incorporation assay. After 10 days of treatment, IGF-I (50 ng/mL) inhibited mineralization by 54±5% (Figure 1B). IGF-I also inhibited the AP activity induced by H_2O_2 (0.8 mmol/L) and TNF-α (10 ng/mL) by 63±6% and 36±9%, respectively (n=4 and n=2, respectively). In addition, IGF-I inhibited LPS (10 ng/mL)-induced alkaline phosphatase activity after 10 days (Figure 1C) and mineralization after 13 days in culture (Figure 1D).

To investigate whether the effects of IGF-I are mediated through IGF-IR, CVCs were treated for 6 days with either IGF-I (25 ng/mL) or [Arg^3]IGF-I (25 ng/mL), which has reduced affinity for IGF binding proteins (IGFBP), or [Leu^24]IGF-I (25 ng/mL), which has reduced affinity for IGF-IR. Results showed that IGF-I and [Arg^3]IGF-I reduced AP activity to 48±2% and 44±6% of control, but [Leu^24]IGF-I did not significantly affect AP activity (96±3% of control). In addition, Northern analysis showed that IGF-IR was expressed in CVCs (Figure 1E).

Intracellular Signaling Mechanisms Mediating IGF-I Inhibition

To investigate the downstream intracellular signaling pathways, CVCs were treated with IGF-I, and activation of the PI3K and ERK pathways were assessed. Western analyses showed that IGF-I activated both protein kinase B (Akt/PKB; downstream target of PI3K) and ERK (Figure 2A). IGF-I did not activate c-Jun N-terminal kinase based on Western analysis (data not shown). To investigate whether both pathways are involved in the inhibitory effect of IGF-I, CVCs were cotreated with IGF-I and either LY294002 (50 μmol/L: PI3K inhibitor) or PD98059 (10 μmol/L: ERK inhibitor), and AP activity was assessed. As shown in Figure 2B, both PD98059 and LY294002 reversed IGF-I inhibition of AP activity, suggesting that the IGF-I inhibitory effect is mediated by both pathways. Treatment of CVCs for 2 days with PDGF (50 ng/mL), which increased the levels of phosphorylated ERK and Akt/PI3K in CVCs, also inhibited AP activity in a similar manner to IGF-I (data not shown).

To investigate the specific effect of the ERK pathway on AP activity, CVCs were transfected with pFC-MEKK (ERK kinase kinase) or mock-transfected and treated with LPS (10 ng/mL) for 2 days. Results showed that LPS-induced AP activity was
analyses were performed using anti-phospho Elk-1 and p90Rsk antibodies. Results showed that Elk-1 was phosphorylated in response to IGF-I (Figure 2A), whereas no phosphorylation was observed with p90Rsk (data not shown).

**Effect of IGF-I on CVC Proliferation**

Because IGF-I is a mitogen, we investigated the effect of IGF-I on CVC proliferation. As shown in Figure 3, CVC proliferation was stimulated by IGF-I after 2 days of treatment. Cotreatment of CVCs with PD98059 (10 μmol/L) or LY29004 (50 μmol/L; LY294002, 50 μmol/L, n = 4).

inhibited by 63% in cells overexpressing MEKK-1 (LPS: 281 ± 65 versus LPS + pMEKK: 101 ± 8, P < 0.005). To investigate further downstream targets of the ERK pathway, Western analyses were performed using anti-phospho Elk-1 and p90Rsk antibodies. Results showed that Elk-1 was phosphorylated in response to IGF-I (Figure 2A), whereas no phosphorylation was observed with p90Rsk (data not shown).

**Discussion**

These findings suggest that IGF-I regulates osteoblastic differentiation and mineralization of calcifying vascular cells, that IGF-I activates both ERK and PI3-Kinase pathways, and that inhibitory effects of IGF-I on osteoblastic differentiation appear to be mediated by both pathways. The findings that either PD98059 or LY294002 can inhibit IGF-I effects suggest that the two pathways share a common downstream target or targets. One such common target is Elk-1, a member of the Ets domain family of transcription factors. Inhibitors of either ERK or PI3K pathways inhibit IGF-I activation of Elk-1 in myogenic cells.13 Interestingly, Elk-1 plays an inhibitory role in maturation and mineralization of osteoblastic cells.14 Our results show that Elk-1 is phosphorylated in response to IGF-I, supporting the role of Elk-1 in IGF-I inhibition of osteoblastic differentiation and mineralization.

Our findings with IGF-I analogs suggest that IGF-I inhibition of CVC differentiation is mediated via IGF-IR, independent of IGFBP. An IGF-I analog with reduced affinity to IGF-IR did not inhibit AP activity, whereas an analog with reduced affinity to IGFBP had an inhibitory effect similar to that of IGF-I. The present findings suggest that IGF-I may regulate vascular calcification by promoting proliferation and inhibiting osteoblastic differentiation of vascular cells. It is also possible that these inhibitory effects may also derive in part from inhibition of apoptosis because apoptosis has been shown to contribute to biomineralization,6 and IGF-I has been shown to block apoptosis.8 Thus, IGF-I may have an important role, not only in osteogenesis in bone, but also in the osteogenic processes leading to vascular calcification.

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**References**


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