Upregulation of IGF2 expression during vascular calcification

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Abstract

The process of vascular calcification shares many similarities with that of skeletal mineralisation and involves the deposition of hydroxyapatite crystals in arteries and cardiac valves. However, the cellular mechanisms responsible have yet to be fully elucidated. In this study, we employed microarray analysis to demonstrate the upregulation of more than 9000 genes during the calcification of murine vascular smooth muscle cells (VSMCs), of which the most significantly, differentially expressed gene was Igf2. Following the validation of increased IGF2 expression by RT-qPCR and immunoblotting in calcifying murine VSMCs, IGF2 expression was further demonstrated in the calcified aorta of the Enpp1<sup>K</sup>/<sup>K</sup> mouse model of medial aortic calcification. Having confirmed that IGF1R and IGF2R were expressed in cultured murine VSMCs, cell-signalling studies in these cells revealed that IGF2 (50 ng/ml) significantly stimulated the phosphorylation of Akt and Erk1/2 (P<0.05). These results potentially indicate that IGF2 may mediate VSMC calcification via the stimulation of Erk1/2 and Akt signalling. This study suggests that the increased IGF2 expression in calcifying VSMCs may reflect the well-established prenatal role of IGF2, particularly as the osteogenic phenotypic transition of VSMCs in a calcified environment recapitulates many of the events occurring during embryonic development. A full understanding of the importance of IGF2 in this pathological process will lead to a better understanding of the aetiology of vascular calcification.

Introduction

The highly conserved insulin-like growth factor (IGF) signalling pathway is unequivocally a major systemic and local (autocrine/paracrine) regulator of growth, controlling cell proliferation and differentiation. The IGF pathway is activated following the binding of IGF1 or IGF2 to IGF type 1 receptor (IGF-R), insulin-receptor and IGF/insulin hybrid-receptor, where it elicits anti-apoptotic, proliferative and metabolic roles (Federici <i>et al.</i> 1997, Belfiore <i>et al.</i> 2009).

IGF2 has a well-established role during embryonic development and is perhaps the most intricately regulated of all growth factors characterised to date (Lee <i>et al.</i> 1990). Its gene is imprinted – only one allele is active, depending on parental origin – and this pattern of expression is maintained epigenetically in almost all tissues. IGF2 activity is further controlled through differential expression of receptors and IGF-binding proteins that determine protein availability (Chao & D’Amore 2008).
This complex and multifaceted regulation emphasises the importance of physiological IGF2 expression and activity.

Deregulation of the IGF2 gene parental imprint results in expression from maternal and paternal alleles, leading to loss of imprinting and IGF2 overexpression. Indeed, the IGF2 gene is rarely transcribed in tissues during postnatal development, with reports of IGF2 gene expression to date limited to tumour cells (Randhawa et al. 1998, Vorwerk et al. 2003, Cui 2007), the choroids plexus and the leptomeninges (DeChiara et al. 1991). Intriguingly, however, data have recently highlighted a crucial role of IGF2 in the regulation of the fate of mesenchymal progenitors during adult bone development and remodelling (Hardouin et al. 2011). Furthermore, IGF2 is critical to the aetiology of human diseases including atherosclerosis, cancer and overgrowth syndromes (Morison et al. 1996, Toretsky & Helman 1996, Eggenschwiler et al. 1997, Sperandeo et al. 2000).

Vascular calcification involves the deposition of hydroxyapatite crystals in blood vessels, valves and cardiac muscle. The pathological process of vascular calcification shares many similarities with that of physiological bone development and formation (Shroff & Shanahan 2007, Demer & Tintut 2008, Zhu et al. 2012). Indeed, numerous reports have demonstrated that vascular smooth muscle cells (VSMCs), the predominant cell type responsible for blood vessel calcification, can undergo phenotypic transition to chondrocytic, osteoblastic and osteocytic cells in a calcified environment (Speer et al. 2005, Zhu et al. 2011). Aortic valve calcification is also believed to involve the differentiation of valvular interstitial cells (VICs) into an osteoblast-like phenotype (Monzack & Masters 2011). Nevertheless, despite intensive investigations, the precise endocrine and molecular signalling mechanisms underpinning this transition have yet to be fully understood.

The revelation of a postnatal role of IGF2 in the survival of progenitor pools in their interactions with the stem cell niches (Hardouin et al. 2011), together with increasing evidence linking IGF2 to vascular function (Chisalita et al. 2009), has led us to examine whether IGF2 is a key mediator of the postnatal phenotypic transdifferentiation of VSMCs during calcification. To our knowledge, this is the first study to investigate the role of IGF2 in the vascular calcification process. In this study, we have carried out in vitro VSMC calcification studies, in conjunction with ex vivo analyses of a mouse model of Generalised Arterial Calcification of Infancy (GACI), to provide the first fundamental insights into the expression profiles of IGF2 during vascular calcification.

**Materials and methods**

**Ethics statement**

All animal experiments were approved by The Roslin Institute’s Animal Users Committee and the animals were maintained in accordance with Home Office guidelines (UK) for the care and use of laboratory animals.

**Maintenance of Enpp1<sup>−/−</sup> mice**

The generation and characterisation of the ectonucleotide pyrophosphatase/phosphodiesterase 1 null (Enpp1<sup>−/−</sup>) mouse has been described previously (Sali et al. 1999, Mackenzie et al. 2012a,b). To determine genotypes, genomic DNA was isolated from ear clips and analysed using PCR protocols developed by Genetyper (New York, NY, USA).

**Primary murine VSMC isolation**

Primary VSMCs were isolated from 5-week-old WT male C57BL/6 mice. Following dissection of the aorta, the adventitia was detached and the blood vessel was opened to expose the endothelial layer of cells (Mackenzie et al. 2011, Zhu et al. 2011, 2013). Aortae isolated from 16 mice were then digested with 1 mg/ml trypsin for 10 min to remove any remaining endothelium and adventitia. This was followed by an overnight incubation at 37 °C in a humidified atmosphere of 95% air/5% CO<sub>2</sub> in growth medium (α-MEM (Invitrogen) supplemented with 10% FCS (Invitrogen) and 1% gentamycin (Invitrogen)). Tissues were then digested in 425 U/ml collagenase type II for 5 h. Before experimental studies, isolated VSMCs were expanded in a growth medium for two passages in T25 tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Baden-Württemberg, Germany) coated with 0.25 g/cm<sup>2</sup> murine laminin (Sigma) to prevent VSMC de-differentiation (Johnson et al. 2008).

**Primary murine VSMC culture**

VSMCs were set down at 1.5×10<sup>4</sup>/cm<sup>2</sup> in 6- or 12-well tissue culture plates containing growth medium. In order to induce calcification of VSMC, Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (P; 3 mM) (Sigma) or β-glycerophosphate (βGP; 2.5 mM) (Sigma) and ascorbic acid (AA; 50 μg/ml) (Sigma) were added to confluent cells for up to 14 days. The cells were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO<sub>2</sub>. The medium was replaced every second/third day.
cDNA microarray hybridisation

RNA was extracted from the cells using RNeasy mini kit (Qiagen Ltd), according to manufacturer’s instructions for microarray analysis. Total RNA content was assessed by absorbance at 260 nm and purity by A260:A280 ratios for each sample. The quality of each sample was considered suitable by a ratio of > 1.9. Target preparation and hybridisation to the Affymetrix Murine 430 2.0 GeneChip were performed by Ark Genomics (The Roslin Institute, University of Edinburgh, UK) following standard Ark Genomics protocols (http://www.ark-genomics.org/protocols). This GeneChip contains ~ 39 000 full-length mouse genes and EST clusters from the UniGene database.

Microarray data analysis

Analysis was carried out as we described previously (Staines et al. 2013). Normalisation across all arrays was achieved using the robust multi-array average expression measure which results in expression measures (summarised intensities) in log base 2 (Irizarry et al. 2003). Comparisons were performed using linear modelling. Subsequently, empirical Bayesian analysis was applied (including vertical (within a given comparison) P value adjustment for multiple testing, which controls for false discovery rate) for which the Bioconductor package limma was used (Kauffmann et al. 2009).

Detection of calcification

Calcium deposition was evaluated through decalcification of the mineralised matrix in 0.6 M HCl for 24 h, and free calcium was determined colorimetrically (Randox Laboratories Ltd, Crumlin, County Antrim, UK) by spectrophotometer (Multiskan Ascent, Thermo Electron Corporation, Vantaa, Finland). The protein content of the cultures was measured and corrected for the following extraction using 1 mM NaOH in 0.1% SDS (Bio-Rad Laboratories Ltd).

RNA isolation and semi-quantitative PCR

Total RNA was extracted from cells using RNeasy total RNA (Qiagen Ltd), according to manufacturer’s instructions. For each sample, total RNA content was assessed by absorbance at 260 nm and purity by A260:A280 ratios. RNA was reverse transcribed and the semi-quantitative PCR was carried out as described previously (Zhu et al. 2011). For the PCR, primers for 18S (Rn18s) rRNA gene (25 cycles) (Ambion, Huntingdon, Cambs, UK, sequence not disclosed), IgfR1 (Fcgr1) and IgfR2 (Fcgr2) (32 cycles) (Qiagen; sequence not disclosed) were used.

Analysis of gene expression using quantitative RT-qPCR

RNA was reverse transcribed and the PCR was carried out as described previously (MacRae et al. 2009). All genes were analysed with the SYBR green detection method using the Stratagene Mx3000P real-time QPCR system (Stratagene, La Jolla, CA, USA). Each sample was run in triplicate. All gene expression data were normalised against glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and the control values were expressed as one to indicate a precise fold change value for each gene of interest. Primers for Runx2 (forward: 5’-ACC ATA ACA GTC TTC ACA ACA CCT-3’, reverse: 5’-CAG GCG ATC AGA GAA CAA ACT A3’), Igf2 (Qiagen; sequence not disclosed) and Gapdh (Primer Design, Southampton, UK; sequence not disclosed) were used.

IGF2 western blotting

The cells were lysed in RIPA buffer (Invitrogen) containing ‘complete’ protease inhibitor cocktail according to manufacturer’s instructions (Roche). Immunoblotting was carried out as described previously (MacRae et al. 2009). Nitrocellulose membranes were probed overnight at 4 °C with anti-IGF2 antibody (1:1000 dilution in 5% BSA), (Abcam, Cambridge, UK) washed in TBST and incubated with anti-rabbit IgG-peroxidase (DAKO, Glostrup, Denmark) for 1 h (1:1000 dilution in 5% milk). The immune complexes were visualised by ECL (GE Healthcare, Buckinghamshire, UK). The membranes were then washed in ‘stripping buffer’ (Pierce, Rockford, IL, USA) and re-probed for 1 h for β-actin expression (1:5000 dilution in 5% milk; anti β-actin clone AC15; Sigma). After washing, the membranes were incubated with anti-mouse IgG-peroxidase for 1 h (Sigma) before ECL detection.

Cell signalling immunoblotting

Following 2 days of culture after confluency, VSMCs were serum deprived for 24 h and then either lysed immediately or stimulated with IGF2 (1–100 ng/ml) for 10–60 min before lysis. The cells were lysed in PhosphoSafe extraction buffer (Merck Biosciences Ltd) containing ‘Complete’ protease inhibitor cocktail as described earlier. The membranes were probed for 1 h at room temperature with primary antibodies raised in rabbit (all 1:1000 dilution in 5% milk) before washing, (forward: 5’-ACC ATA ACA GTC TTC ACAACA CCT-3’, reverse: 5’-CAG GCG ATC AGA GAA CAAACT A3’), Igf2 (Qiagen; sequence not disclosed) and Gapdh (Primer Design, Southampton, UK; sequence not disclosed) were used.
dilution in 5% milk) against phospho-Akt (ser 473), total Akt, phospho-P44/42 Map kinase (Thr202/Tyr204) and total P44/42 Map kinase (Cell Signalling Technology, Beverly, MA, USA). The membranes were then incubated with anti-rabbit IgG-peroxidise (Cell Signalling Technology) for 1 h (1:1000 dilution in 5% milk). The immune complexes were visualised as described earlier. Assessment of band intensity was achieved using Quantity One image analysis software (Bio-Rad Labs, Inc.).

**Immunohistochemistry of Enpp1<sup>−/−</sup> murine aorta**

Aortae were dissected from 22-week-old Enpp1<sup>−/−</sup> and WT mice. After fixation in 10% neutral buffered formalin, the tissues were finally dehydrated and embedded in paraffin wax before sectioning at 4 μm using standard procedures (Zhu et al. 2011). For histological analysis, sections were de-waxed in xylene and antigen retrieval was achieved by treatment of sections with 0.1% trypsin for 10 min. Endogenous peroxidises and non-specific antibody binding were blocked before overnight incubation at 4°C with 0.5 μg IgG/ml anti-IGF2 (Abcam). The slides were then washed in PBS and incubated with goat anti-rabbit IgG peroxidise (1:200 dilution) using the Vectastain ABC kit (Vector Laboratories, Peterborough, UK) following manufacturer’s instructions. The sections were then dehydrated, counterstained with haematoxylin and eosin and mounted in DePeX. The control sections were incubated with non-immune rabbit IgG (0.5 μg IgG/ml) in place of the primary antibody.

**Statistical analysis**

General linear model analysis and the Student’s t-test were used to assess the data. All data are expressed as the mean ± S.E.M. Statistical analysis was performed using Minitab 16. P < 0.05 was considered to be significant.

**Table 1** Genes exhibiting the greatest differential expression (log fold change) in murine VSMCs cultured for 9 days in calcifying conditions

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>(Log) Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Igf2</td>
<td>Insulin-like growth factor 2</td>
<td>4.13</td>
<td>1.21×10⁻¹⁴</td>
</tr>
<tr>
<td>Ramp1</td>
<td>Receptor activity modifying protein 1</td>
<td>4.01</td>
<td>1.21×10⁻¹⁴</td>
</tr>
<tr>
<td>Sept4</td>
<td>Septin 4</td>
<td>2.57</td>
<td>3.24×10⁻¹³</td>
</tr>
<tr>
<td>Trib3</td>
<td>Tribbles homologue 3</td>
<td>-2.92</td>
<td>3.47×10⁻¹³</td>
</tr>
<tr>
<td>Art4</td>
<td>ADP-ribosyltransferase 4</td>
<td>2.05</td>
<td>1.08×10⁻¹²</td>
</tr>
<tr>
<td>Trib3</td>
<td>Tribbles homologue 3</td>
<td>-2.47</td>
<td>1.75×10⁻¹²</td>
</tr>
<tr>
<td>Rasgrp2</td>
<td>RAS, guanyl-releasing protein 2</td>
<td>2.35</td>
<td>1.75×10⁻¹²</td>
</tr>
<tr>
<td>Tnem204</td>
<td>Transmembrane protein 204</td>
<td>3.44</td>
<td>4.35×10⁻¹²</td>
</tr>
<tr>
<td>Sept4</td>
<td>Septin 4</td>
<td>2.24</td>
<td>4.35×10⁻¹²</td>
</tr>
<tr>
<td>Gpr116</td>
<td>G protein-coupled receptor 116</td>
<td>2.57</td>
<td>1.57×10⁻¹¹</td>
</tr>
</tbody>
</table>

**Results**

**Global transcriptome profiling reveals Igf2 upregulation in calcifying VSMCs**

In order to identify novel mediators of vascular calcification, microarray analysis was performed on murine VSMCs cultured under calcifying conditions. Analysis using Bioconductor limma software demonstrated that following normalisation, 957 genes were upregulated and 472 genes were downregulated following 9 days of culture (n = 4, greater than twofold change). These data revealed that the embryonic developmental gene Igf2 exhibited the greatest differential expression in calcifying VSMCs (Table 1). The full data obtained from the hybridisation comparisons can be viewed on the 6 EMBL-EBI Array Express repository (http://www.ebi.ac.uk/microarray-as/aer/?#aemain[0]) (accession no. MTAB-1680).

Furthermore, gene expression analysis by qRT-PCR validated the key finding of Igf2 enhancement. As previously demonstrated by our laboratory and others (Murshed et al. 2005, Huang et al. 2008, Li et al. 2008, Zhu et al. 2013), high phosphate (Pi) induced a significant increase in VSMC mineralisation as quantified by calcium deposition (Fig. 1A) after day 7 and 14 days in the culture. Associated with this increase in VSMC mineralisation, Igf2 mRNA expression was also significantly increased at the same time points in culture (P<0.001; Fig. 1B). This increased Igf2 transcript expression was ratified at the protein level (Fig. 1C), with densitometry quantification revealing an increase in protein expression in cells cultured under calcifying conditions at day 7 (mean = 1.81-fold; S.E.M. = 0.38) and day 14 (mean = 2.96-fold; S.E.M. = 0.34). Together, these data confirm the upregulation of Igf2 expression in our microarray data. Interestingly, IGF2 protein expression in VSMCs appeared to reduce with time in culture (Fig. 1C), which may be
associated with altered interactions of IGF-binding proteins with IGF2. A significant increase in mRNA expression of the osteogenic marker Runx2 ($P < 0.001$) was also seen at 7 and 14 days, indicating that the transition to the osteoblast phenotype was in progress and thereby validating this in vitro model to study VSMC calcification (Fig. 1D).

Expression of IGF2 is associated with aortic calcification in a mouse model of GACI

The association of IGF2 with vascular calcification was further strengthened by examination of the Enpp1$^{-/-}$ mouse, a recognised murine model of GACI. This mouse model of the frequently lethal disease GACI presents with decreased levels of the mineralisation inhibitor, inorganic pyrophosphate (PPi), and phenotypic features including significant alterations in bone mineralisation in long bones and calvaria, and pathologic, severe perisphinal soft tissue and moderate medial arterial calcification (Sali et al. 1999, Mackenzie et al. 2012a,b). Calcification in the medial layer of the Enpp1$^{-/-}$ aorta was confirmed by alizarin red staining (Fig. 2A), with no mineralisation deposits observed in WT controls (Fig. 2B). IGF2 was immunolocalised strongly to Enpp1$^{-/-}$ calcified aortic media (Fig. 2C and D) but present only weakly in the aorta of WT mice (Fig. 2E and F). Control sections in which the primary IGF2 antibody was substituted by non-immune rabbit IgG were shown to be negative (Fig. 2G and H). The identification of increased IGF2 expression in mineralising aortae of Enpp1$^{-/-}$ mice supports our microarray and in vitro VSMC data, confirming our novel finding that the upregulation of IGF2 is strongly associated with the vascular calcification process.

IGF2 activates Akt and Erk1/2 signalling in murine VSMCs

To understand the molecular pathways initiated by IGF2 in vascular tissue, we first determined the profile of IGF receptors expressed in murine VSMCs. Expression of both IGF1R and IGF2R was confirmed in cultured murine VSMCs and interestingly expression levels of these
receptors were not altered with the addition of phosphate (Fig. 3A). Signal transduction studies were next performed to disclose the signalling mechanism(s) by which IGF2 may modulate VSMC calcification. Akt and Erk1/2 phosphorylation were both significantly ($P < 0.05$) induced following IGF2 treatment at 50 and 100 ng/ml (Fig. 3B, C, and D), potentially indicating that IGF2 may mediate VSMC calcification via stimulation of Erk1/2 and Akt signalling.

IGF2 does not directly modulate VSMC calcification

Having established the increased expression of IGF2 during the VSMC calcification process, we sought to establish whether IGF2 promotes or inhibits vascular calcification by direct treatment of VSMCs with recombinant IGF2. Intriguingly, IGF2 treatment did not impact on VSMC calcification in vitro (Fig. 4). These data suggest that the generation of IGF2 during vascular calcification does not actively regulate this pathological process. Rather, the increased IGF2 expression in calcifying VSMCs may reflect a phenotypic transition to an embryonic phenotype.

Discussion

The IGF system consists of two ligands, IGF1 and IGF2, which share >60% sequence identity. A series of elegant in vitro and in vivo studies have highlighted a key role of IGF1 in mediating vascular calcification (Radcliff et al. 2005, Siddals et al. 2011). Our work expands the current knowledge of the role of IGF2 in disease. In this study, we provide the first evidence to suggest that IGF2 also contributes to this pathological process.

Despite recent advances in our knowledge, the full mechanisms underpinning vascular calcification are unknown. This study has clearly demonstrated differential expression of novel genes in calcifying murine primary aortic VSMC cultures. Microarray analysis, functional enrichment, qRT-PCR and immunoblotting validation studies together identified for the first time, the upregulation of IGF2 during in vitro VSMC calcification. These in vitro data were confirmed and extended by studying an in vivo mouse model of vascular calcification. Mice lacking NPP1, a major generator of extracellular pyrophosphate (ePPi), spontaneously develop articular cartilage, perispiral and medial aortic calcification at a young age.
These *Enpp1*−/− mice share phenotypic features with a human disease, idiopathic infantile arterial calcification (Rutsch et al. 2001, 2003). In this study, our immunohistochemical approach demonstrated increased expression of IGF2 in the calcified media of *Enpp1*−/− aortic tissue. This data add to a body of increasing evidence linking IGF2 to vascular function (Kim et al. 1998, Chisalita et al. 2009). In particular, *Igf2* mRNA is upregulated in the aortas of *Apoe*−/− mouse model of atherosclerosis (Zaina et al. 2002), a disease commonly accompanied by aortic calcification. In addition, it has been suggested that IGF2 may participate in angiogenesis (Kim et al. 1998), a process necessary for vascular calcification to occur (Collett & Canfield 2005).

In order to elucidate the potential mechanism through which IGF2 may be modulating VSMC calcification, changes in the PI3-kinase/Akt and MAPK/Erk1/2 signalling pathways were examined. These pathways are involved in a wide range of cellular processes, including growth, transcription, proliferation, migration, survival, differentiation and calcification (Roy et al. 2001, Salasznyk et al. 2004, Kok et al. 2009, Zhu et al. 2012). Previous studies have shown that IGF2 increases Erk1/2 and Akt phosphorylation in a range of tissue types, including chondrocytes, hepatocytes and skeletal myocytes (Hamamura et al. 2008, Liang et al. 2010, Bower et al. 2012). Our data revealed that IGF2 induced both the phosphorylation of Akt and Erk1/2 in murine VSMCs.
confirming the recent report describing IGF2-induced activation of these pathways in human aortic smooth muscle cells (Chisalita et al. 2009).

In this study, we show for the first time an increase in IGF2 expression in calcifying VSMCs. However, whilst recombinant IGF2 treatment did not impact on VSMC calcification in vitro, the increased expression may reflect the well-established prenatal role of IGF2. In particular, the phenotypic transition of VSMCs to a bone-like phenotype in a calcified environment recapitulates many of the events occurring during embryonic development. This is further supported by the recent demonstration that Igf2 directs the fate of mesenchymal progenitors during bone development and remodelling (Hardouin et al. 2011). A full understanding of the importance of IGF2 in this pathological process will lead to a better comprehension of the aetiology of vascular calcification and may offer novel therapeutic strategies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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