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Role of Carbonic Anhydrase 2 (CAR2) in Ectopic Calcification

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Abstract

Introduction—Osteopontin (OPN) is a potent inhibitor of ectopic calcification. Previous studies suggested that, in addition to blocking apatite crystal growth, OPN promoted regression of ectopic calcification by inducing the expression of acid-generating carbonic anhydrase II (CAR2) in monocyte-derived cells.

Methods—To test this hypothesis, OPN and CAR2 expression and calcification of subcutaneously implanted glutaraldehyde fixed bovine pericardium (GFBP) were studied in CAR2 mutant mice.

Results—Consistent with previous studies in Black Swiss mice, GFBP calcified to a greater extent in OPN^{-/-} mice compared to OPN^{+/+} mice on the C57Bl/6 background. GFBP implanted in CAR2 deficient mice (CAR2^{-/-}) were significantly more calcified than those implanted into wildtype mice (CAR2^{+/+}) (37 + 5 versus 20 + 6.5 ug Ca/mg tissue, respectively at 30 days ($p < .001$), and 42 + 5 versus 20 + 4 ug Ca/mg tissue at 60 days, respectively ($p < .001$)). On the other hand, OPN levels within and surrounding the implants were similar in CAR2^{+/+} and CAR2^{-/-} mice, suggesting that OPN expression in the absence of CAR2 was not sufficient to mitigate ectopic calcification.

Conclusions—These results indicate that CAR2 expression is an important regulator of ectopic calcification, potentially by facilitating OPN mediated mineral regression.

Keywords

Carbonic anhydrase II; Osteopontin; ectopic calcification

Introduction

Ectopic calcification, or soft tissue mineralization, occurs in a number of pathological settings and is a major cause of bioprosthetic and native cardiac valve failure. Evidence of bone cell gene expression coupled with findings in mouse knock-out models have begun to redefine ectopic calcification as a cell-mediated process involving a large repertoire of inductive and inhibitory molecules [1]. In this context, osteopontin (OPN) has emerged as a potent inhibitor of ectopic calcification.

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OPN, an acidic phosphoprotein, is normally found in mineralized tissues such as bone and is thought to regulate mineralization by inhibiting apatite crystal growth and promoting osteoclast function via the $\alpha_v\beta_3$ integrin [2-4]. While OPN expression in soft tissues is much more limited, our group and others have reported that OPN is abundant at sites of ectopic calcification including human atherosclerotic plaques and calcified aortic valves [5, 6], and several lines of evidence suggest that OPN is an endogenous inhibitor of ectopic calcification. In vitro, addition of exogenous osteopontin inhibits vascular smooth muscle cell calcification [7, 8] and smooth muscle cell cultures derived from OPN null mice calcify their matrices to a greater extent than wildtype mice [9]. In addition, matrix gla protein null mice that develop spontaneous vascular calcification [10] demonstrate even greater vascular calcification when bred onto the OPN null background [11]. Finally, using a subcutaneous implantation model, it was shown that glutaraldehyde-fixed vascular implant materials (porcine aortic valve leaflets and bovine pericardium) calcify to a much greater extent in OPN deficient mice (OPN^{-/-}) compared to wild-types (OPN^{+/+}) [12, 13].

Although the precise anticalcific mechanism of action of OPN is unclear, histological analysis of GFBP implant materials in OPN^{+/+} mice showed co-localization of OPN and calcium deposits and suggested a mechanism involving physical inhibition. Furthermore, heterozygotes for the OPN deficiency (OPN^{+/-}) showed early onset of GFBP calcification followed by subsequent regression of calcification. Significantly, and relevant to the aim of the current work, regression was highly correlated with the levels of carbonic anhydrase II (CAR2) expressing monocyte-derived cells and the subsequent local acidification of the implants [12]. Finally, GFBP pre-coated with OPN showed less mineralization and enhanced CAR2 levels following subcutaneous implantation compared to control GFBP [13]. Together, these studies suggested that OPN acts not only as an inhibitor of vascular calcification through direct physical inhibition of crystal growth, but also through the promotion of active regression via a mechanism involving CAR2. Thus, to further test the hypothesis that CAR2 is an important regulator of ectopic calcification, in the present study GFBP calcification was examined in CAR2 mutant mice.

Materials and Methods

Animals

OPN mutant mice were generated in a 129/SvJ $\times$ Black Swiss background as previously described [14]. Hybrid mutant mice were backcrossed onto the C57Bl/6 background for greater than 10 generations. OPN homozygous wildtype (OPN^{+/+}) and null (OPN^{-/-}) mice on a C57Bl/6 background were used in these studies.

CAR2-deficient mice (CAR2^{-/-}) on a C57BL/6 background were generated and genotyped using breeders obtained from Jackson Laboratories (Bar Harbor, ME). The creation of the CAR2^{-/-} mouse is described in detail elsewhere [15]. The *Car2ⁿ* null allele has a C to T transition in the CAA codon for Gln¹⁵⁴ that results in a termination codon near the beginning of exon 5 yielding premature termination of translation. CAR2 cannot be detected in *Car2ⁿ* homozygotes and expression is reduced to one-half in heterozygotes.

Implant Material

Glutaraldehyde-fixed (0.625%) bovine pericardium (GFBP) tissue was a gift from Edwards Lifesciences (Irvine, CA).

GFBP subcutaneous implantation model

Animals were housed and used in specific-pathogen-free (SPF) facilities according to the protocol approved by the Institutional Animal Care Use Committee (IACUC) at the University of Washington. Male and female mice between 6 and 8 weeks of age were selected for subcutaneous implantation of GFBP tissue. OPN mutant mice were anesthetized by IP injection with a mix of Xylazine (0.01 mg/g mouse) and Ketamine (0.15 mg/g mouse) in saline. The carbonic anhydrase II deficient mice (CAR2^{-/-}) used in this study are in a state of metabolic acidosis brought on by their gene deficiency [16]. Depressed ventilation due to anesthesia during implantation surgery compounds this state by inducing an additional respiratory acidosis, which is marked by the inability of mice to adequately exchange CO₂. Therefore we used a reduced concentration of a standard anesthetic mixture (ketamine/xylazine in saline from previous experiments) to minimize the acidity introduced to the system. The dosage of Xylazine/Ketamine was 0.008 mg/0.13mg/g of mouse. In addition each mouse was intubated and ventilated to compensate for the reduction in gas exchange during implant surgery and recovery. An endotracheal tube [Intramedic Polyethylene Tubing #427420 with a 0.034 × 0.050 inch (I.D. × O.D.) and a 2.cm length with beveled end] was inserted into the trachea (approximately 2-3mm) and connected to a small animal ventilator (North American Drager, model Narkomed II-A, Telford; tidal volume: 0.50-0.60 mL, rate: 105-115 rpm). Circular GFBP implants, made using sterile 8 mm diameter biopsy punches (Miltex Instrument Co. Inc., Lake Success, NY), and rinsed in PBS, were implanted subcutaneously in the backs of mice (two implants per animal). At appropriate time-points, mice were euthanized by IP injection of Nembutal (400 uL/ animal). Implants were removed with an intact foreign body capsule, cut in half and used for calcium quantitation and histological staining respectively. For calcium quantitation, the foreign body capsule was removed from explanted GFBP tissue and samples were stored at -80°C until calcium assay. At all time points, the foreign body capsule separated easily and cleanly from the GFBP. For histological staining, explanted GFBP tissue, with an intact foreign body capsule was fixed overnight in methyl carnoys (3:1, methanol: acetic acid) prior to histological processing. National Institutes of Health (NIH) guidelines were observed for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985). The time-course of calcification was determined for subcutaneously implanted GFBP tissue in OPN mutant mice at 7, 14, 30, and 60 days and in CAR2 mutant mice at 14, 30 and 60 days.

Calcium quantitation

Implant calcium quantitation was performed as previously described [12]. Briefly, explanted GFBP tissues were lyophilized overnight, weighed and incubated in 0.6 N HCl at 37°C for 24 hours. The calcium content of the 0.6 N HCl supernatant was determined with a modified colorimetric method (Calcium Assay Kit, Teco). The total calcium content of each tissue section was normalized with regard to tissue dry weight. The final implant calcium quantitative value was an average of the two implants per animal.

Implant Histological analysis

Fixed GFBP tissue explants were dehydrated and embedded in paraffin. Hematoxylin and eosin (H&E) differential staining was used on 5µm sections, staining nuclei of cells bluish-purple and other cellular elements pink respectively. Alizarin Red S staining was used to detect mineralization in 5 µm sections, as previously described. Immunostaining was performed on adjacent sections with macrophage-specific rat anti-mouse BM-8 antibody (Accurate Chemical & Scientific Corp., Westbury, NY), goat anti-mouse Osteopontin (OPN) affinity purified polyclonal antibody (R&D Systems, Inc., Minneapolis, MN), and sheep anti-human Carbonic Anhydrase II (CAR2) antibody (Biodesign International, Kennebunk, ME).

Statistical Analysis

Data is presented as means $\pm$ SEM of triplicate measures analyzed using simple ANOVA. Post hoc tests were conducted using Fisher's LSD protected t-tests. Differences were considered significant at p-values of < 0.05 and are noted as such.

Results

GFBP implantation studies

We previously found a significant difference in the mineralization of GFBP starting at day 7 following subcutaneous implantation in OPN^{-/-} vs OPN^{+/+} mice on a calcification-susceptible Black Swiss genetic background [13]. In the present study, mice on the calcification-resistant C57Bl/6 mouse strain were utilized since the CAR2 mutation was available in this background. Mice are known to be susceptible to ectopic calcification in a strain dependent manner [17, 18]. Hence, we first established the time course of ectopic calcification of GFBP in wildtype and OPN^{-/-} mice on the C57Bl/6 background. As shown in figure 1, GFBP calcified to a greater extent in OPN^{-/-} mice compared to OPN^{+/+} mice in C57Bl/6 mice as expected, but this effect was significant only after 60 days of implantation. Furthermore, the maximum level of calcification in OPN mutant mice on the C57Bl/6 background was significantly lower than similar GFBP implant experiments performed on a Black Swiss background at all time points examined (data not shown). These studies confirmed the anticalcific effect of OPN, and established a time line to study GFBP calcification in CAR2 mice on the relatively calcification resistant C57Bl/6 background.

To determine the role of CAR2 in ectopic calcification, GFBP were implanted into wildtype and CAR2^{-/-} mice. As shown in Figure 2, CAR2^{-/-} mice showed significantly greater calcification compared to wildtype mice at 30 and 60 days following subcutaneous implantation. These studies suggest that CAR2, similar to OPN, acts as an inhibitor of ectopic calcification in this model system.

Implant Histological analysis

Using histological analysis, mineralization and the expression pattern of CAR2 and OPN surrounding GFBP implants were determined. At 30 days post-implantation, very little mineralization was observed in GFBP explanted from CAR2^{+/+} mice by alizarin red S staining (Figure 3a), consistent with calcium quantitation data (Figure 2). Similar to

previous studies [12, 13], abundant CAR2 positive cells were found in the foreign body reaction surrounding the GFBP implant (Figures 3b and 3c). Expression of CAR2 was predominantly found in monocyte-derived macrophages and foreign body giant cells as determined by staining with BM8 (Figure 3b and 3c, and data not shown). OPN staining overlapped with CAR2 positive cells, and was also associated with calcified deposits (data not shown), identical to previous studies [12, 13]. No immunoreactivity for CAR2 was observed in CAR2^{-/-} mice (Figure 3d) confirming the specificity of the anti-CAR2 antibody.

In contrast to CAR2^{+/+} mice, GFBP implanted into CAR2^{-/-} mice for 30 days showed significant calcification by alizarin red S staining (Figure 4b). Discrete calcified foci were observed within the GFBP, but not in the surrounding foreign body reaction. As expected, no immunoreactivity for CAR2 was observed in or around the GFBP in CAR2^{-/-} mice (Figure 3d, and data not shown). However, a high level of OPN immunoreactivity was observed both within the GFBP, associated with calcified foci, and in cells in the surrounding foreign body reaction (Figure 4c). BM8 staining identified these cells as monocyte-derived macrophages and giant cells (data not shown). Consistent with previous studies [12], high levels of OPN staining were observed in association with foreign body giant cells surrounding GFBP implants in CAR^{-/-} and CAR^{+/+} mice (Figure 4c and 4d). By H&E staining, no difference in the overall morphology of the GFBP implant or amount of surrounding foreign body reaction was observed in CAR2^{-/-} mice compared to CAR2^{+/+} mice (Figure 4a, and data not shown). These data indicate that OPN expression, in the absence of CAR2 activity, is insufficient to mitigate GFBP calcification in this model.

Discussion

The goal of the current study was to characterize the ectopic calcification phenotype of CAR2^{-/-} mice using an *in vivo* GFBP implantation model. The major finding of this study was that subcutaneously implanted GFBP calcified to a greater extent in CAR2^{-/-} mice compared to CAR2^{+/+} mice, indicating an important anticalcific role for this enzyme in the regulation ectopic calcification. Moreover, OPN expression in CAR2^{-/-} and CAR2^{+/+} mice was comparable, suggesting that the ability of OPN to act as an inhibitor of ectopic calcification depends on CAR2 activity.

Carbonic anhydrases are vital in the maintenance of acid-base homeostasis in living organisms. CAR2, more specifically, is a cytosolic enzyme expressed at varying levels in a wide array of cell types including monocyte-derived macrophages, foreign body giant cells, and osteoclasts [12, 13, 19]. CAR2 catalyzes the reversible hydration of CO₂ and is critical for proper ion exchange and acid/base balance [16]. In osteoclasts, CAR2 facilitates proton production and leads to the acidification of resorption lacunae and ultimately the dissolution of bone [20]. Consistent with *in vitro* findings, individuals with CAR2 deficiency display inhibited bone resorption and an osteopetrotic phenotype in addition to renal acidosis [21]. While mice and humans with CAR2 deficiency show metabolic acidosis, it is unlikely that this is responsible for the observed enhanced susceptibility to ectopic calcification since acidity increases the solubility of calcium phosphate salts, and thus would favor decreased calcification rather than the observed enhanced calcification in CAR2 null mice.

Our data suggest a direct role for CAR2 in regulating ectopic calcification in addition to skeletal biomineralization and acid-base balance. These findings are consistent with previous studies showing that CAR2^{-/-} mice show an age dependent susceptibility to vascular calcification primarily in arterioles and small arteries in addition to an osteopetrotic phenotype [19]. In our model system, CAR2 was expressed in macrophages and giant cells of the foreign body reaction, suggesting that these cells had taken on osteoclast-like properties. On the other hand, Essalihi et al., using a rat model of warfarin and vitamin K1-induced arterial medial calcification [22, 23], showed that endothelin receptor antagonists induced mineral regression through the activation of smooth muscle membrane bound carbonic anhydrase IV (CA IV). In their model system, OPN and CAR2 were also abundant at calcified regions of the aorta and appeared to limit the extent of calcification. Initial attempts to detect the presence of CA IV expression localized to our implants were negative (data not shown). However, this does not eliminate the possibility that both CAR2 and CA IV are important in the mitigation of ectopic vascular calcification. Both CAR2 and CA IV may indeed play a predominant role in the dissolution of ectopic mineral that is dependent on the pathologic condition. This finding would be in agreement with redundant roles of carbonic anhydrases shown in other physiologic functions.

In conclusion, we have demonstrated that GFBP calcify to a greater extent in CAR2^{-/-} mice compared to CAR2^{+/+} mice, despite equivalent levels of OPN accumulation. These findings indicate that in our subcutaneous implantation calcification model, maximal mitigation of ectopic calcification requires both OPN and CAR2. Although it is possible that CAR2 may act either at the level of mineral resorption and/or at the level of mineral formation, we believe the former is more likely based on its cellular localization. Future studies to more directly address the relationship between Car2 and OPN including development of a Car2^{-/-} × OPN^{-/-} double knockout mouse and analysis of implant calcification in this model system are needed.

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Summary

Ectopic calcification is an actively regulated process involving inductive, inhibitory and regressive mechanisms. The current studies indicate that carbonic anhydrase II is involved in regulating ectopic calcification, most likely as a downstream mediator of osteopontin-induced mineral regression.

Figure 1. Time-course of calcification of GFPB in OPN^{-/-} and OPN^{+/+} mice on C57BL/6 background
Quantitation of calcium levels in explanted GFPB at 14, 30, and 60 days post-implantation respectively. Bars indicate standard error of the mean.

Figure 2. Time-course of calcification of GFBP in CAR2-/- and CAR2+/+ mice

Quantitation of calcium levels in explanted GFBP at 14, 30, and 60 days post-implantation respectively. Bars indicate standard error of the mean.

Figure 3. Histological analysis of GFBP subcutaneously implanted into CAR2+/+ mice

GFBP were explanted from CAR2+/+ mice after 30 days and stained with a) alizarin red S or b, c) antibody directed against CAR2. Arrows identify CAR2 positive macrophages and giant cells. d) As a control, GFBP from CAR2-/- mice were stained with antibody directed against CAR2. Dashed lines demarcate the interface between the GFBP (P) and the host foreign body reaction.

Figure 4. Histological analysis of GFBP subcutaneously implanted into CAR2^{-/-} mice
 GFBP were explanted from CAR2^{-/-} (a, b and c) after 30 days and stained with a) hematoxylin and eosin, b) alizarin red S, or c) antibody directed against OPN. d) As a control, GFBP from CAR2^{+/+} mice were stained with antibody directed against OPN.
 Dashed lines demarcate the interface between the GFBP (P) and the host foreign body reaction.