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Original Article

Role of carbonic anhydrase II 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-deficient mice compared to wild types on the C57Bl/6 background. GFBP implanted in CAR2-deficient mice (CAR2$^{-/-}$) were significantly more calcified than those implanted into wild-type mice (CAR2$^{+/+}$) [37±5 vs. 20±6.5 μg Ca/mg tissue, respectively, at 30 days (*P*<.001), and *42*±5 versus *20*±4 μg 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. © 2009 Elsevier Inc. All rights reserved.

Keywords: Carbonic anhydrase II; Osteopontin; Ectopic calcification

1. 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 knockout 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.

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 OPN 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 wild-type 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 colocalization of OPN and calcium deposits and suggested a mechanism involving

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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 precoated 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.

2. Materials and methods

2.1. Animals

OPN mutant mice were generated in a 129/SvJ X Black Swiss background, as previously described [14]. Hybrid mutant mice were backcrossed onto the C57Bl/6 background for greater than 10 generations. $OPN^{+/+}$ and $OPN^{-/-}$ 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, USA). The creation of the $CAR2^{-/-}$ mouse is described in detail elsewhere [15]. The *Car2*^{n} null allele has a C-to-T transition in the CAA codon for Gln^{154} that results in a termination codon near the beginning of exon 5, yielding premature termination of translation. CAR2 cannot be detected in *Car2*^{n} homozygotes, and expression is reduced to one half in heterozygotes.

2.2. Implant material

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

2.3. GFBP subcutaneous implantation model

Animals were housed and used in specific-pathogen-free facilities according to the protocol approved by the Institutional Animal Care Use Committee 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 intraperitoneal injection with a mix of xylazine (0.01 mg/g mouse) and ketamine (0.15 mg/g mouse) in saline. The CAR2-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 CO_2. 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-in. (I.D.×O.D.) and a 2-cm length with beveled end] was inserted into the trachea (~2–3 mm) 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, Lake Success, NY, USA) and rinsed in phosphate-buffered saline 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 μl/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.

2.4. 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 h. 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.

2.5. Implant histological analysis

Fixed GFBP tissue explants were dehydrated and embedded in paraffin. Hematoxylin and eosin (H&E)

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