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Cartilage-derived retinoic acid-sensitive protein (CD-RAP): A stage-specific biomarker of heterotopic endochondral ossification (HEO) in fibrodysplasia ossificans progressiva (FOP)

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ABSTRACT

Background: Genesis of a cartilaginous scaffold is an obligate precursor to bone formation in heterotopic endochondral ossification (HEO). We tested the hypothesis that cartilage-derived retinoic acid-sensitive protein (CD-RAP) can serve as a plasma biomarker for the pre-osseous cartilaginous stage of HEO. Palovarotene, a retinoic acid receptor-gamma (RARγ) agonist, has been proposed as a possible treatment for fibrodysplasia ossificans progressiva (FOP) and is a potent inhibitor of HEO in mouse models. Current drug development for FOP mandates the identification of stage-specific biomarkers to facilitate the evaluation of clinical trial endpoints.
Results: Here we show in an injury-induced, constitutively-active transgenic mouse model of FOP that CD-RAP levels peaked between day-7 and day-10 during the zenith of histologically-identified chondrogenesis, preceded radiographically apparent HEO, and were diminished by palovarotene. Cross-sectional analysis of CD-RAP levels in plasma samples from FOP patients demonstrated a statistically non-significant trend toward higher levels in the recent flare-up period (three weeks to three months within onset of symptoms). However, in a longitudinal subgroup analysis of patients followed for at least six months after resolution of flare-up symptoms, there was a statistically significant decrease of CD-RAP when compared to levels in the same patients at the time of active or recent exacerbations.
Conclusions: These data support the further exploration of CD-RAP as a stage-specific biomarker of HEO in FOP.

1. Introduction

In the mature adult, new skeletal formation is normally restricted to regeneration of bone at sites of fracture. However, heterotopic endochondral ossification (HEO), or the formation of extraskeletal bone through a cartilage template can occur in response to musculoskeletal or central nervous system (CNS) injury [1,2], or in the rare genetic disease fibrodysplasia ossificans progressive (FOP) [3,4]. FOP, the most devastating form of HEO, is caused by heterozygous activating missense mutations in activin receptor IA/activin-like kinase 2 (ACVR1/ALK2) [5]. In FOP, episodic exacerbations (flare-ups) result in HEO causing ankyloses of axial and appendicular joints rendering movement impossible [3,4]. In both non-hereditary HEO and in FOP, trauma and inflammation are major precipitating factors for the development of ectopic bone [6–13].

Clinically, early symptoms of lesion formation that result in HEO are nonspecific and manifest as pain, soft tissue swelling and periarticular stiffness [1,13–17]. Signs are also nonspecific and may include erythema, warmth, swelling, and tenderness, with progressive decreased range of motion and possible joint ankylosis as later findings [15,17]. Although complete bone maturation may take at least 6 months, late signs and symptoms usually occur by 12 weeks and may be clinically evident as early as two to three weeks after injury [9,18].

Radiographically, the presence of heterotopic bone lags behind the clinical presentation of an evolving lesion. At initial presentation, there may be little or no evidence of bone formation on plain radiographs. However, as a lesion evolves through its penultimate stages, bone formation becomes evident on plain radiographs [19]. Radiographic findings of osteogenesis may take five to eight weeks to appear after the first symptoms begin. Radionuclide bone scans may suggest the presence of HEO lesions at earlier stages prior to their appearance on plain radiographs [19]. Radionuclide bone scans show positive uptake during

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early vascular and late bone phases of HEO, exhibit increased activity for up to two years after the onset of HEO and are non-specific. Serum alkaline phosphatase can be elevated late in lesion formation, usually plateaus by eight weeks, and declines afterward, but is not always associated with the kinetics or severity of HEO [20,21]. Thus, there are no reliable biomarkers to predict that HEO will occur before radiographic evidence of ectopic ossification is detected.

Palovarotene, a retinoic acid receptor-gamma (RARγ) agonist, has been proposed as a possible treatment for fibrodysplasia ossificans progressiva (FOP) and is a potent inhibitor of obligate chondrogenesis in HEO in mouse models of FOP [22]. Current drug development for FOP mandates the identification of stage-specific biomarkers to facilitate the evaluation of clinical trial endpoints [23]. Here we tested the hypothesis that cartilage-derived retinoic acid-sensitive protein (CD-RAP), a protein expressed during chondrogenesis, can serve as a plasma biomarker for the pre-osseous phase of HEO.

2. Methods

2.1. Animal model and palovarotene treatment

A transgenic mouse model containing a constitutively active (ca)ALK2 allele flanked by loxP sites (caALK2 mice; caQ207D) was used in all animal experiments [10–13,24–26]. A 50 μl, 0.9% NaCl solution containing adenovirus-Cre [5×10^{10} genome copies (GC) per mouse; Penn Vector Core, University of Pennsylvania] to induce expression of caALK2, and cardiotoxin (100 μl of a 10 μM solution; Sigma-Aldrich, St. Louis, MO) to induce an injury/inflammatory response, was injected into the hindlimb musculature of mice at 23 days of age. CaALK2 mice were treated via gavage with 1.47 mg/kg/day or 2.94 mg/kg/day of palovarotene [22] in a vehicle containing 2.9% DMSO in corn oil, or with 10 μl/g of vehicle control. Mice were treated for 4 days prior to and for 2, 5, 7, 10, or 14 days following injection in the left hind limb. Blood and other tissues were recovered at 0, 2, 7, 10, and 14 days after injections. For the control arm 4 mice were used at each time point 0, 2, 5, 7, and 10 days post-induction. A total of 16 mice were collected at 14 days post-induction and a random sampling of 8 was analyzed for CD-RAP levels. For palovarotene 1.47 mg/kg, 8 mice were collected at day 14. For palovarotene 2.94 mg/kg, 3 mice were collected at day 2, 4 mice at day 5 and 7, 5 mice at day 10 and 9 mice at day 14 post-induction.

2.2. Patient samples

Blood samples were obtained from FOP patients according to a protocol approved by the University of Pennsylvania Institutional Review Board. Flare-ups were defined by clinical symptoms (e.g., pain, swelling, decreased movement, stiffness, warmth, or redness) as documented in the patient's medical record at the time of blood collection. The time of onset of a flare-up was informed by patient or caregiver history documented in the medical record at the time of blood collection. Patients were assigned to one of three clinical categories based on disease activity at the time of blood collection: **active flare-up** (those with active flare-ups within 3 weeks of blood collection); **recent flare-up** (those with a history of flare-up between 3 weeks and 3 months of blood collection); and **no recent flare-up** (those with no disease activity for at least six months). The patient age range was 14 to 42 years. Blood collection was obtained from patients of both sexes.

2.3. Histology

Mouse hind limbs were excised promptly from every mouse following blood collection by cardiac puncture. Four mice per time point underwent limb excision for histology. Mouse hind limbs were excised, cleaned of soft tissue, and fixed in 3.7% formaldehyde for 72 h. Isolated bone and muscle tissue was dehydrated in graded alcohols (70 to 100%), cleared in xylene and embedded in paraffin. Paraffinized tissue blocks were cut into 5 μm sections using a Polycut-S motorized microtome (Reichert-Jung, Nossloch, Germany). The sections were stained with 0.1% Safranin O and counterstained with 0.05% Fast Green by standard methods. Limb sections were visualized for HEO formation using a Nikon Eclipse 90i microscope and Nikon Plan Fluor 20× objective (Nikon Inc., Melville, New York, USA). Representative images were captured using NIS Elements Imaging Software 3.10 Sp2 and a DS Ri1 camera.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Plasma from mice was assayed for CD-RAP level using the mouse MIA ELISA kit from CUSABIO (Wuhan, Hubei Province, China) according to the manufacturer's instructions. Patient plasma was assayed for CD-RAP level using the human MIA Quantikine ELISA kit from R&D Systems (Minneapolis, MN, USA) following manufacturer instructions.

2.5. Micro-computerized tomography

Micro-computed tomography (μCT) was performed on the injected leg post-mortem using a Scanco VivaCT 40 (Bruettisellen, Switzerland) to determine the volume of heterotopic bone and obtain a two-dimensional image of the medial view of the sagittal plane of the limb. Scanning was performed using a source voltage of 55 kV, a source current of 142 μA, and an isotropic voxel size of 10.5 μm. Bone was differentiated from "non-bone" by an upper threshold of 1000 Hounsfield units and a lower threshold of 150 Hounsfield units.

2.6. Functional wire grasp test

Mobility in the left hind limb was assessed in mice 14 days post-injury by having mice climb on a wire. Unimpaired mice have the ability to grasp the wire with all four limbs while mice without any mobility in a limb can only grasp the wire with three limbs.

2.7. Statistics

One-way or two-way ANOVA with Tukey-Kramer post-hoc analysis was used to compare the effects of palovarotene, or palovarotene and time, respectively, on levels of CD-RAP. Cross-sectional data from patient samples were also analyzed by ANOVA. For longitudinal data from patient samples, the two-tail, dependent *t*-test for paired samples was used to assess the mean difference between the active/recent flare up group and the no recent flare-up group. The level of statistical significance was set to $p < 0.05$. All analyses were performed using GraphPad software (www.graphpad.com).

3. Results

3.1. Injury induces HEO in caALK2 mice and is inhibited by the RARγ agonist palovarotene

In an injury-induced transgenic mouse model of FOP-like HEO (caALK2) 100% of animals formed extra-skeletal bone by 14 days after injury (Fig. 1A and B, no drug controls). In the presence of 1.47 mg/kg and 2.94 mg/kg doses of retinoic acid receptor (RAR) gamma (RARγ) agonist (palovarotene) there were statistically significant reductions in HEO (Fig. 1A and B). These doses of palovarotene represent the human dose equivalent of 5 mg/day and 10 mg/day, respectively; the doses currently being used in phase 2 clinical trials (ClinicalTrials.gov; Identifier: NCT02190747). Concomitant with reductions in HEO, animals receiving palovarotene retained mobility about the injured limb (Fig. 1C).

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