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Elevated plasma RANTES in fibrodysplasia ossificans progressiva – A novel therapeutic target?

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ABSTRACT

Fibrodysplasia ossificans progressiva (FOP) is a rare hereditary disease caused by a mutation in the intracellular domain of the activin A receptor type I and is characterized by episodes (flare-ups) of progressive heterotopic endochondral ossification (HO) in the soft tissues. The mutation alone is not sufficient for the occurrence of HO since flare-ups are triggered by inflammation and activation of the innate immune system. A number of cellular and humoral mediators have been implicated in animal and *in vitro* models. Observations in humans support the inflammatory nature of the condition, but data on the involved mediators are variable. We hypothesize that for induction of flare-ups in patients with FOP increase in at least one of the pro-inflammatory cytokines is both essential and sufficient to trigger the entire process of the inflammatory cells influx resulting in the novel ectopic bone formation and we suggest that C–C motif ligand 5 (CCL5), a pro-inflammatory chemokine also known as Regulated on activation, normal T-cell expressed and secreted (RANTES), might be the key candidate. CCL5 is a chemoattractant for all cellular types implicated in HO and is produced by the cells of the tissue micro-environment at the sites of HO as well as by the pro-inflammatory cellular mediators. CCL5 induces ossification in cultured human pluripotent mesenchymal cells (hMSCs) and in the primary culture of monocytes from FOP patients (but not from their healthy relatives), stimulation with lipopolysaccharide induces CCL5 expression. Finally, in a pilot study we used a panel of 23 cytokines and chemokines to screen the plasma samples of three subjects: a female patient with FOP during a flare-up; a female patient with *hyperostosis corticalis generalisata* (van Buchem disease), another rare disease characterized by excessive bone formation at the sites where it regularly occurs that does not include inflammatory events; and a healthy woman without bone disorders. There appeared a rather clear-cut signal of a 2-fold higher level of CCL5 in the FOP patient vs. the healthy subject and the van Buchem patient. Evaluation of the hypothesis would require an international prospective study, with main motivation being the lack of a conclusive treatment as the major unmet need in FOP. A treatment targeting CCL5 receptor already exists and is used in HIV-infected patients.

Abbreviations: RANTES, regulated on activation, normal T cell expressed and secreted; FOP, Fibrodysplasia ossificans progressive; HO, heterotopic ossification; CCL2, 5, 7, 8, C-C motif ligand 2, 5, 7, 8; BMP, bone morphogenetic protein; ACVR1, activin A receptor type I; G-CSF, granulocyte colony-stimulating factor; TNF-α, -β, tumor necrosis factor alpha, beta; IL-1α, -2, -3, -5, -6, -7, -8, -10, -13, -15, interleukin-1α, -2, -3, -5, -6, -7, -8, -10, -13, -15; NK, natural killer; CCR1, 3, 5, 7, C–C chemokine receptor type 1, 3, 5, 7; hMSC, human mesenchymal stem cells; LPS, lipopolysaccharide; CD14, cluster of differentiation 14; CXCL1, 2, 3, 9, 10, C-X-C motif chemokine 1, 2, 3, 9, 10; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; HIV, human immunodeficiency virus; NIH, National Institutes of Health; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; TGFβ1, transforming growth factor beta 1

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Introduction

Fibrodysplasia ossificans progressiva (FOP) is a rare hereditary disease characterized by a progressive heterotopic endochondral ossification (HO) in soft tissues. Its clinical course is variable with episodes marked by bone neo-formation (*flare-ups*) that can be induced by medical procedures, trauma, infections, or can occur without any obvious trigger. There is no conclusive therapy for the disease and an effective treatment is a crucial unmet need [1]. FOP is caused by a mutation in the intracellular domain of the bone morphogenetic protein (BMP) type I receptor – activin A receptor type I (ACVR1), however, the molecular and pathophysiological mechanisms of disease progression in relation to the mutated ACVR1 and its ligands are still unknown [2,3]. It is generally accepted that the mutation alone is not sufficient for the occurrence of HO and that postnatal FOP flare-ups are induced by inflammation and activation of the innate immune system [4]. Data from *in vitro* and animal models have repeatedly suggested involvement of different cell types (progenitor cells, various immunocytes), proinflammatory cytokines and local reaction of the immune system and tissue microenvironment components in this complex process [5]. For example, depletion of macrophages or depletion and inhibition of mast cells in mutated Acvr1 mice reduced HO and proinflammatory cytokine expression at the site of the soft tissue injury [6,7]. The link between inflammatory biomarkers and HO formation in humans with FOP is less clear. A study in 15 FOP patients (cases) and 25 of their healthy relatives (controls) indicated no apparent difference in serum concentrations of 25 pro-inflammatory cytokines between them, although in two patients with flare-ups, granulocyte-colony stimulating factor (G-CSF) and tumor necrosis factor alpha (TNF-α) levels tended to be higher than in controls [8]. Another study [9] suggested that patients with FOP even without flare-ups display a pro-inflammatory state: serum concentrations of several pro-inflammatory interleukins, i.e., IL-3, IL-7, IL-8, IL-9, and of tumor necrosis factor (TNF) α were higher in 7 patients with no flare-ups than in their heathy relatives (n = 6). However, concentration of the anti-inflammatory IL-10 was also higher [9], while levels of these cytokines in 6 FOP patients with flare-ups did not differ from the control values [9]. Only the levels of the granulocyte-macrophage colony stimulating factor (GM-CSF) were higher in FOP patients with a flare-up compared to healthy controls [9].

The hypothesis

We hypothesize that for induction of heterotopic ossification (a flare-up) in patients with FOP increase in at least one pro-inflammatory cytokine is both essential and sufficient to trigger the entire process of inflammatory cell influx with already known role and function in osteogenesis (Fig. 1). If identified (and readily obtainable), such a cytokine might serve as a marker to monitor the disease phase, guide decisions about potential therapies or itself be a target for a novel therapeutic approach. While (relative) contributions of different cytokines might somewhat vary between individual patients, we hypothesize that C–C motif ligand 5 (CCL5), a pro-inflammatory chemokine, also known as Regulated on activation, normal T-cell expressed and secreted (RANTES), might be the key candidate.

Observations in support of the hypothesis

Literature data

CCL5 belongs to the C–C chemokine subfamily that induces the *in vitro* migration and recruitment of T cells, natural killer (NK) cells, mast cells, eosinophils and basophils. It is produced by T lymphocytes, macrophages, platelets, fibroblasts, endothelial and epithelial cells [10] and is involved in angiogenesis, tumor growth, fibrosis, and recruitment of inflammatory cells to the sites of injury and infection [11–13]. CCL5 binds to its receptor CCR5, but also to CCR1 and CCR3 [14,15]. In the study by Liu et al. [16] CCL5 and its receptor CCR1 were crucial and sufficient for osteogenesis in human mesenchymal stem cells (hMSCs), and CCL5 promoted osteogenesis as indicated by the increased alkaline phosphatase activity. In the study by Barruet et al. [9], lipopolysaccharide (LPS) treatment of cluster of differentiation 14+ (CD14 +) primary monocytes isolated from FOP patients with (n = 6) or without (n = 6) a flare-up (but not in the samples from healthy controls, n = 16) resulted in increased CCL5, CCR7 and C-X-C motif chemokine 10 (CXCL10) mRNA levels with a prolonged activation of the nuclear factor kappa light chain enhancer of the activated B cells (NF-κB) (a transcription factor regulating a range of pro-inflammatory cytokines).

Empirical data – a pilot study

Outline

We used a panel of 23 pro- and anti-inflammatory cytokines and chemokines to screen the plasma samples of three subjects: (i) a female patient with FOP during a flare-up and no corticosteroid treatment. Blood sample was taken as a part of a regular work-up during hospitalization taking care not to induce HO by the needle insertion; (ii) an otherwise healthy female patient with *hyperostosis corticalis generalisata* (van Buchem disease), a rare bone disease with a different pathological basis, characterized by excessive bone formation at the sites where it regularly occurs that does not involve inflammatory processes. Blood sample was taken during a regular check-up; and (iii) a healthy woman without bone disorders (blood sample taken at a regular check-up). All subjects (36–44 years of age) provided informed consents for the use of their sample for the present analysis.

Materials and methods

Blood samples were drawn into syringes containing 3.8% sodium citrate to form an anticoagulant-to-blood ratio (v/v) 1:9. Plasma was obtained by centrifugation (15 min at 4 °C and 3000 g), and aliquots of each sample were stored at −80 °C until analysis. Human cytokine antibody arrays (#ab133996, Abcam) were performed using patient plasma samples (diluted 1:3) according to the manufacturer's manual and analyzed using the Chemi Doc imaging system (BioRad) according to manufacturer's recommendation. Background correction and quantification of fluorescent signals was performed with ImageJ 1.52a software (NIH) [17]. Plasma cytokine expression levels (in technical duplicates) were log-transformed and analyzed in a linear mixed-model. Fold difference of disease vs. healthy control sample was obtained with 95% confidence intervals.

Results

In line with the fact that each of the three clinical conditions was represented by only a single subject, plasma expression levels of a number of cytokines in the FOP and the van Buchem disease patients somewhat differed from those in the healthy subject. In agreement with a previous report [8], G-CSF levels tended to be higher in the FOP patient than in the healthy woman (Figs. 2 and 3), however, there appeared a rather clear-cut signal of a 2-fold higher level of CCL5. Others [9] have reported higher serum expression levels of several interleukins – IL-3, IL-7, IL-8 and IL-10 – in FOP patients without a flare (but not in those with a flare) than in healthy controls. We observed a tendency towards higher IL-7 and lower IL-3, IL-8, IL-10 and IL-15 plasma levels in our patient at a flare-up as compared to the healthy subject (Fig. 2). Expression levels of several cytokines in the van Buchem disease patient apparently deflected from the “healthy” levels, all towards the lower values (Fig. 2) and do not support any specific interpretation except that they are in line with the non-inflammatory nature of the disease.

Consequences of the hypothesis and discussion

To our knowledge, this is the first indication of increased plasma

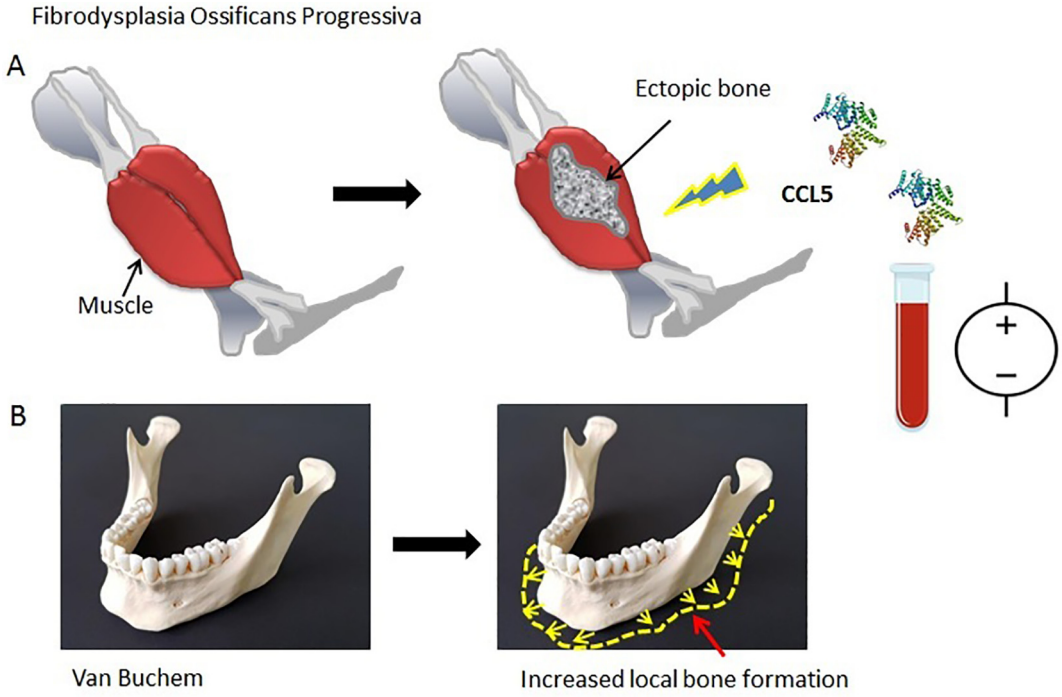

Fig. 1. Hypothesis. The induction of heterotopic ossification (a flare-up) in patients with FOP (A) could be result of increased amount of in at least one pro-inflammatory cytokine essential and sufficient to trigger the entire process of inflammatory cell influx with already known role and function in osteogenesis. This makes it distinct compared to the other rare bone diseases that involve different disease pathology background (B).

CCL5 in a patient with FOP during a flare-up suggested by the contrast vs. the levels in a healthy subject and a subject with a different (non-inflammatory) bone disorder characterized by excessive ossification. It is limited by the fact that it was observed in only a single patient – the only one of the three FOP patients in the country (a small Central European country with a population of around 4 millions) who consented to blood donation. A similar signal of higher CCL5 vs. a concurrent healthy female volunteer was observed for this same patient on two previous flare-up occasions, but data are not shown since blood samples underwent hemolysis. The present pilot is also limited by the fact that CCL5 levels in this patient during periods without a flare-up have not been determined: she did not consent to blood donation being afraid that venipuncture could provoke it. However, when taken together with the *in vitro* observations of the CCL5 effect in the hMSCs culture (16) and the CCL5 expression response to LPS stimulation in the primary FOP patients' monocyte culture [9], the present pilot supports a role of CCL5 in FOP. Replication of this observation in other patients and in other countries would justify a larger international prospective study of CCL5 in pathophysiology of FOP. CCL5 could serve as a potential biomarker to e.g., monitor FOP flare-ups, and more importantly, CCL5 and its receptors could be considered as a potential therapeutic target: currently, the only approved CCL5-targeting treatment is used in human immunodeficiency virus (HIV) infected patients, but CCL5 as a target has been extensively considered in cancer [18] and inflammation [10].

Declaration

Ethics approval and consent to participate

Blood samples from all three subjects were taken as a part of routine scheduled procedures – diagnostic work-up in the FOP and van Buchem patient, and during a regular check-up in the healthy volunteer. All three subjects provided informed consents for the use of their samples for the present analysis.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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CRediT authorship contribution statement

Lovorka Grgurević: Conceptualization, Writing - original draft. **Ruđer Novak:** Formal analysis, Writing - review & editing. **Vladimir Trkulja:** Data curation. **Lejla Ferhatović Hamzić:** Conceptualization, Writing - review & editing. **Stela Hrkač:** Writing - review & editing. **Simeon Grazio:** Resources, Writing - review & editing. **Marija Santini:** Resources, Writing - review & editing.

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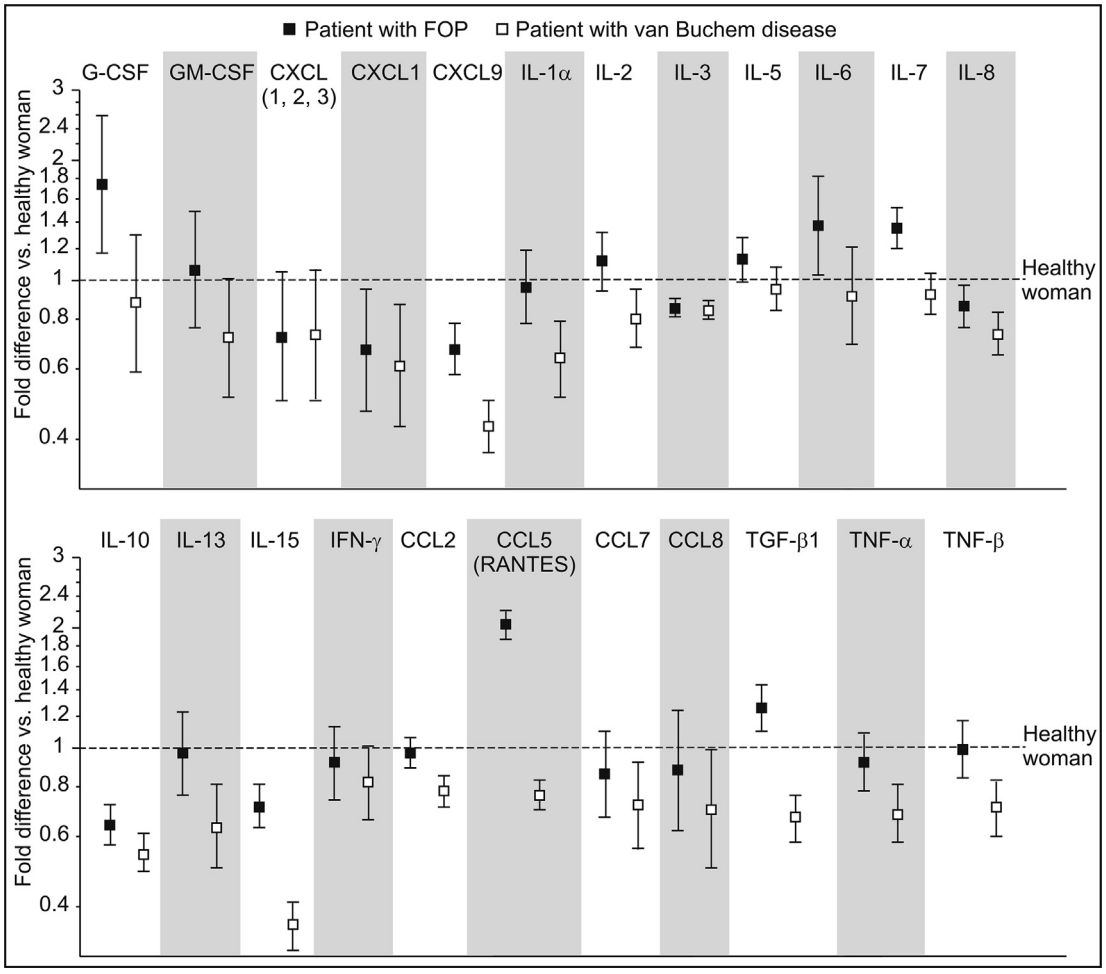

Fig. 2. Cytokine expression. Expression (as fold difference) of various cytokines in plasma of a patient with *fibrodysplasia ossificans progressiva* (FOP) and a patient with *hyperostosis corticalis generalisata* (van Buchem disease) relative to the healthy control subject (depicted as dashed horizontal line indicating the reference value = 1.0). Log-transformed data obtained in technical duplicates were analyzed in a linear mixed-model and differences (i) FOP – control and (ii) van Buchem disease – control were exponentiated to obtain a ratio, i.e., "fold-difference" (closed and open squares) vs. control with 95% confidence intervals (bars).

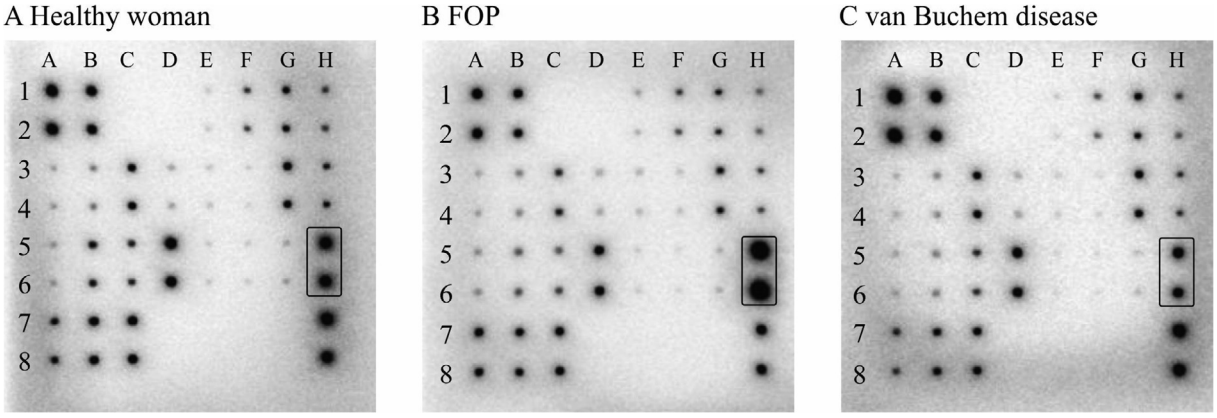

Fig. 3. Cytokine array membranes. Cytokine expression profiles of a healthy woman (A), fibrodysplasia ossificans progressiva (FOP) patient (B) and hyperostosis corticalis generalisata (van Buchem disease) patient (C) plasma samples. A1, A2, B1, B2, H7, H8 = positive control; C1, C2, D1, D2 = negative control; E1, E2 = G-CSF; F1, F2 = GM-CSF; G1, G2 = CXCL(1,2,3); H1, H2 = CXCL1; A3, A4 = IL-1a; B3, B4 = IL-2; C3, C4 = IL-3; D3, D4 = IL-5; E3, E4 = IL-6; F3, F4 = IL-7; G3, G4 = IL-8; H3, H4 = IL-10; A5, A6 = IL-13; B5, B6 = IL-15; C5, C6 = IFN-γ; D5, D6 = CCL2; E5, E6 = CCL8; F5, F6 = CCL7; G5, G6 = CCL9; H5, H6 = CCL5 (black rectangle); A7, A8 = TGF-β1; B7, B8 = TNF-α; C7, C8 = TNF-β; D7, D8, E7, E8, F7, F8, G7, G8 = blank. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

"Determination of the differences in the protein profile and incidence of inflammatory mediators in the blood and urine of patients with FOP, and bone induction assay by using the egg extract in cell culture" and by the Scientific Center of Excellence for Reproductive and Regenerative Medicine (project "Reproductive and regenerative medicine – exploration of new platforms and potentials", Grant Agreement KK01.1.1.01.0008 which is funded by the European Union through the European Regional Development Fund.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mehy.2019.109313.

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