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Inhibition of TGFβ signaling decreases osteogenic differentiation of fibrodysplasia ossificans progressiva fibroblasts in a novel in vitro model of the disease☆

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ABSTRACT

Fibrodysplasia ossificans progressiva is a rare genetic disorder characterized by progressive heterotopic ossification. FOP patients develop soft tissue lumps as a result of inflammation-induced flare-ups which leads to the irreversible replacement of skeletal muscle tissue with bone tissue. Classical FOP patients possess a mutation (c.617G>A; R206H) in the *ACVR1*-encoding gene which leads to dysregulated BMP signaling. Nonetheless, not all FOP patients with this mutation exhibit equal severity in symptom presentation or disease progression which indicates a strong contribution by environmental factors. Given the pro-inflammatory role of TGFβ, we studied the role of TGFβ in the progression of osteogenic differentiation in primary dermal fibroblasts from five classical FOP patients based on a novel method of platelet lysate-based osteogenic transdifferentiation. During the course of transdifferentiation the osteogenic properties of the cells were evaluated by the mRNA expression of Sp7/Osterix, Runx2, Alp, OC and the presence of mineralization. During transdifferentiation the expression of osteoblast markers Runx2 ($p < 0.05$) and Alp were higher in patient cells compared to healthy controls. All cell lines exhibited increase in mineralisation. FOP fibroblasts also expressed higher baseline Sp7/Osterix levels ($p < 0.05$) confirming their higher osteogenic potential. The pharmacological inhibition of TGFβ signaling during osteogenic transdifferentiation resulted in the attenuation of osteogenic transdifferentiation in all cell lines as shown by the decrease in the expression of Runx2 ($p < 0.05$), Alp and mineralization. We suggest that blocking of TGFβ signaling can decrease the osteogenic transdifferentiation of FOP fibroblasts.

1. Introduction

1.1 Fibrodysplasia ossificans progressiva (FOP) is an extremely debilitating disease leading to gradual ossification of skeletal muscles and other soft tissues [1]. The prevalence of FOP is only one in two million people and there are no identified ethnic, gender or geographical factors that affect its occurrence [2]. The course of the disease starts with the congenital malformation of the great toes and its progression is episodic depending on the appearance of flare-ups which give rise to heterotopic ossification (HO) lesions in the body. The flare-ups can be spontaneous, trauma- or influenza-induced and are characterized by inflammation, soft tissue swelling, and pain with variable duration and severity [3,4]. A commonly shared characteristic of FOP heterotopic ossification (HO) is the type of tissues it inflicts which include skeletal muscles, tendons, ligaments, fascia and aponeuroses. Similarly, there are also specific tissue types which are never involved in this disease such as the

Abbreviations: FOP, fibrodysplasia ossificans progressiva; FCS, fetal calf serum; BMP, bone morphogenetic protein; TGFβ, transforming growth factor β; Runx2, runt-related transcription factor 2; Alp, alkaline phosphatase; OC, osteocalcin; ACVR1, activin receptor IA; ALK2, activin-like kinase 2; HO, heterotopic ossification; SHED, stem cells from human exfoliated deciduous teeth; iPSC, induced pluripotent stem cells; NSAID, nonsteroidal anti-inflammatory drug.

☆ Conflicts of interest: none.

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diaphragm, tongue, extra-ocular muscles, skin as well as the cardiac and smooth muscles [5].Tissue ossification cannot be reversed and it eventually leads to severe physical immobility [6]. Available treatment can only target pain and inflammation during symptom exacerbation [7].

1.2 FOP shows an autosomal dominant pattern of inheritance caused in the majority of cases by a mutation (c.617G>A; R206H) in the glycine and serine (GS)-rich domain of activin receptor IA (ACVR1), also known as activin-like kinase 2 (ALK2) [8]. This leads to dysregulated bone morphogenetic protein (BMP) signaling by producing a mutant form of the receptor with enhanced activity [9,10]. BMPs are protein molecules with diverse function which are important in the control of cell fate during the transition of mesenchymal cells into the osteogenic cell lineage [11]. They function by forming a complex with two BMP type II receptors and two BMP type I receptors. In this way BMP type I receptors become activated by the phosphorylation of serine residues in the GC domain which leads to the dissociation of the inhibitory FKBP12 protein from the GS domain of ACVR1 and the activation of Smad1, 5 and 8 which, in turn, bind to Smad4 in order to convey downstream signaling to the nucleus for the transcriptional regulation of target genes. The FOP mutation can disturb the interaction of FKBP12 with the GS domain which diminishes its inhibitory effect [9] leading to the extensively documented enhanced BMP signaling effect in FOP patients [12–15] and animal models [16].

1.3 Despite the identification of the 100% penetrant *ACVR1* mutations, FOP patients exhibit great variability in the progression and severity of the disease which highlights the significance of environmental factors. Three pairs of monozygotic twins with FOP showed identical congenital great toe malformation but the postnatal progression of the disease followed a different course based on environmental exposure [17]. It has been long established that environmental triggers that generate inflammation such as intramuscular vaccinations, mandibular blocks in dental treatments, influenza infections and trauma events can induce flare-ups and subsequent FOP progression [4,18]. Minimizing exposure to these factors prevents rapid deterioration of the disease [7]. The involvement of inflammation is demonstrated by the infiltration of mononuclear inflammatory cells in early FOP lesions prior to skeletal muscle destruction [19]. In addition, inflammatory mast cell density has been found to be higher in FOP lesional tissue [20]. Moreover, an inflammatory stimulus was required for HO in a FOP mouse model with inducible expression of constitutive active ALK2 receptor [21] and in a BMP4-overexpressing mouse model of HO [22].

1.4 Transforming growth factor β (TGFβ) is a multifunctional cytokine regulating a plethora of cellular processes such as inflammation, migration, differentiation, morphogenesis, cell cycle arrest and apoptosis in a cell type- and signal-dependent manner [23,24]. TGFβ responsiveness is ubiquitous in mammalian cells [25]. Similarly to BMP, it also belongs to the TGFβ superfamily and as such it also controls cellular functions by forming a complex with a tetrameric receptor consisting of two type II (TGβR-II) and two type I (TGβR-I) receptors. This activates Smad2 and Smad3 which regulate the expression of TGFβ-responsive genes following their association with Smad4 [23]. TGFβ has a central role in osteoblast differentiation and bone tissue generation [26] which has been exemplified in numerous transgenic mouse models. TGFβ can also crosstalk with the Wnt, parathyroid hormone (PTH) and fibroblast growth factor (FGF) to modulate skeletal development [27]. TGFβ has also been shown to have a broad immunopathological role which can depend on the context of cell microenvironment including the presence of other immunomodulatory factors [28,29]. Thus, it is likely to be involved in inflammation-induced flare-ups.

1.5 We aimed to study the role of TGFβ in flare-ups-induced ossification. Therefore, we generated a novel in vitro model of osteogenic transdifferentiation based on the use of primary dermal fibroblasts from the FOP patients. Growth factor-induced osteogenic transdifferentiation was performed by platelet lysate-based osteogenic media. Platelet lysate is a rich source of growth factors including TGFβ and BMPs [30,31]. It is shown to induce osteogenic differentiation of human mesenchymal cells [32,33] whereas osteogenic transdifferentiation of fibroblasts with platelet-rich plasma has been demonstrated in mice [34]. Although the culprit cell type responsible for FOP remains elusive, BMP signaling has been suggested to alter stem cell fate [22]. Given the lack of specialized fibroblast-specific markers [35], their ability to differentiate into other cell types [36], the identification of different populations of stem cells in the epidermis [37], and the presence of highly fibroproliferative lesions in FOP patients [20], fibroblasts are appropriate for the study of FOP flare-ups and osteogenic cell differentiation.

1.6 In this experimental system we investigated the role of TGFβ in promoting osteogenic transdifferentiation of FOP fibroblasts based on the expression of the osteoblast specific markers Sp7/Osterix, runt-related transcription factor 2 (Runx2), alkaline phosphatase (Alp) and osteocalcin (OC) and in vitro mineralisation. We compared the osteogenic potential of FOP and transdifferentiated control cells. The role of TGFβ was probed pharmacologically with the TGFβ type I receptor inhibitor GW788388.

2. Methods

2.1. Cell culture

A 3 mm full thickness skin biopsy was carefully taken under local infiltration anesthesia with 1 ml phosphate-buffered lidocaine solution by an experienced dermatologist. This procedure allowed for minimized local trauma and prevented the generation of local fibrotic reactions. The skin biopsy was immediately placed in Ham F10 media supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Life Technologies). On the same day the biopsy was finely sectioned with a scalpel. The tissue sections were placed in a T25 tissue culture flask (ThermoScientific) and allowed 2 h at room temperature to attach. This was followed by the addition of 2 ml of cell culture media and placing in a humidified atmosphere with 37 °C and 5% CO_2. In weeks 2–3 fibroblast cell growth was observed and the cell culture media was increased to 5 ml. Cells were routinely stored in liquid nitrogen after resuspending in complete Ham F10 media with 10% DMSO.

2.2. Clinical evaluation of FOP patients

The clinical characteristics of the patients were evaluated at the endocrinology section of the VU University Medical Center hospital by an experienced specialist (Table 1). All patients presented their first flare-up during the first 4 years of their life. The number of flare-ups of the last year was recorded (based on swelling and/or pain and treated in collaboration with a doctor). At the time of biopsy, the patients were questioned for present flare-ups and number of present affected joints. Patients 2, 4 and 5 received medication due to a local flare-up which was administered from at least 1 week prior to biopsy acquisition till at least one week thereafter. Patients 2 and 4 received nonsteroidal anti-inflammatory drugs (NSAIDs) and although some effect was noted, the flare-up was still active during biopsy acquisition. Patient 5 received pain relief medication. During biopsy acquisition the patients received no corticosteroids or extra other medication while daily contact with the involved specialist was ensured. No flare-ups were recorded at the site of biopsy acquisition. No existing flare-ups exacerbated and no new flare-ups developed after the biopsy was performed. The tissue at the biopsy site healed within a few days leaving hardly any scar and there was no sign of heterotopic ossification as a result of the biopsy up to one year later.