Involvement of Thrombin and Osteopontin in the pathophysiology of Dupuytren's Contracture

Iino, T; Tsujii, M; Hasegawa, M; Uemura, T; Kokubu, N; Satonaka, H; Asanuma, K; Sudo, A

Mie University, Tsu, Japan

Introduction: Myofibroblast, a specialized type of fibroblast, plays an important role in the pathology for Dupuytren's contracture (DC) due to their ability of the production of the contractile force that is involved in the contracture [1]. Moreover, inflammatory infiltration in palmer fascia of DC has been reported by several authors, who concluded that DC may be a chronic inflammatory disease. Thrombin is a multi-functional serine protease and a key enzyme of blood coagulation, catalyzing the conversion of fibrinogen to fibrin. Additionally, thrombin is a potent inducer of fibrogenic cytokines, such as TGF-β, connective tissue growth factor and ECM proteins such as collagen, fibronectin, and tenascin-C in various cells [2]. Osteopontin (OPN), an extracellular matrix glycoprotein, can also modulate a variety of cellular activities associated with chronic inflammatory disease, including proliferation, adhesion and surviva. In fact, recent studies showed that OPN was expressed in various fibrotic conditions including myocardial fibrosis after ischemic heart disease, renal fibrosis, liver cirrhosis and lung fibrosis [3, 4]. Additionlly, the presence of the thrombin-cleaved form of OPN is well correlated with various inflammatory disease activities. We herein presented the expression of osteopontin (OPN) and thrombin-cleaved OPN in myofibroblast of DC and upregulation of α-smooth muscle actin (αSMA)'s and OPN's expression after application of thrombin to the cultured cells from Dupuytren's fascia.

Methods: Subjects: The study group consisted of 25 patients (4 women and 21 men) who underwent resection of the palmer fascia for DC. The patients' mean age was 69.1 years (range, 58 to 82 years). All patients signed an informed consent document, and the study was approved by the institutional review board. The palmer apponeurosis resected in carpal tunnel release were used as control.
Immunohistochemical analysis: The specimens were performed on serial sections with monoclonal mouse anti-αSMA antibody (Dako), anti-OPN antibody (O-17, IBL), and anti-OPN N-half (34E3, IBL). After visualization, immunolabed images were provided on the screen of a computer and morphometric analysis was performed using Lumina Vision version 1.11 software (Mitani Shoji Co.). A total of 4 fields in each specimen were evaluated for staining area as the percentage of expressing areas divided by the total area. The expression areas for αSMA and OPN were also evaluated by fluorescent double-immunostaining. The secondary antibody for visualization of αSMA and OPN were used green emission for Alexa Fluor 488 and red for Alexa Fluor 546.
Cell culture: The cells of nodules and cord in Dupuytren's fascia from 5 different patients were minced with a scalpel under sterile conditions and then incubated in F-12 Ham's medium containing 10% fetal bovine serum, streptomycin (100 mg/ml), 300 units/ml

collagenase and 5000 units/ml dispase at 37°C for 12 hours. Isolated cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and streptomycin (100 mg/ml). For the determination of the effect of thrombin, the Dupuytren's cells were incubated in DMEM containing 1 U/ml thrombin (Mochida Pharmaceutical Co.). After 72 hours, total proteins were collected from cells.
Western blotting: Immunostaining was performed using antibodies directed against αSMA and OPN (O-17) and an ECL blotting detection agent (GE healthcare Bio-Science Co.).
Statistical analysis: StatView 5.0 for Windows software (SAS Institute, NC, USA) was used for statistical analysis. Data were analyzed using Spearman's coefficient of lank correlation. Values of $p<0.05$ were deemed statistically significant.

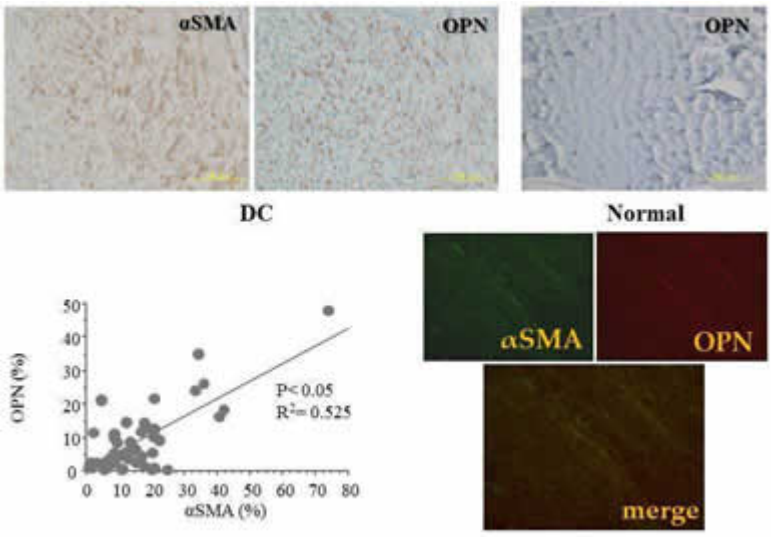
Results: The expression of OPN was in the nodules of fascia and appeared to be similar pattern to that of αSMA except the wall of vessels (Fig. 1A). In fact, expression's area of these two molecules was significantly correlated (Fig. 1B). Additionally, fluorescent double- immunostainig clearly showed the co-localization between OPN and αSMA (Fig. 2). Furthermore, thrombin-cleaved OPN (34E3) was also immunelabeled on similar areas with OPN (O-17) in nodules of Dupuytren's fascia (Fig. 3), considered that the majority of OPN's expression was thrombin-cleaved form in the nodules centered in the pathology. After treatment of thrombin, there were clear upregulation of expression of αSMA and OPN in the cells from nodules as well as cord (Fig 4), although there were weak expression of these molecules without application of thrombin.

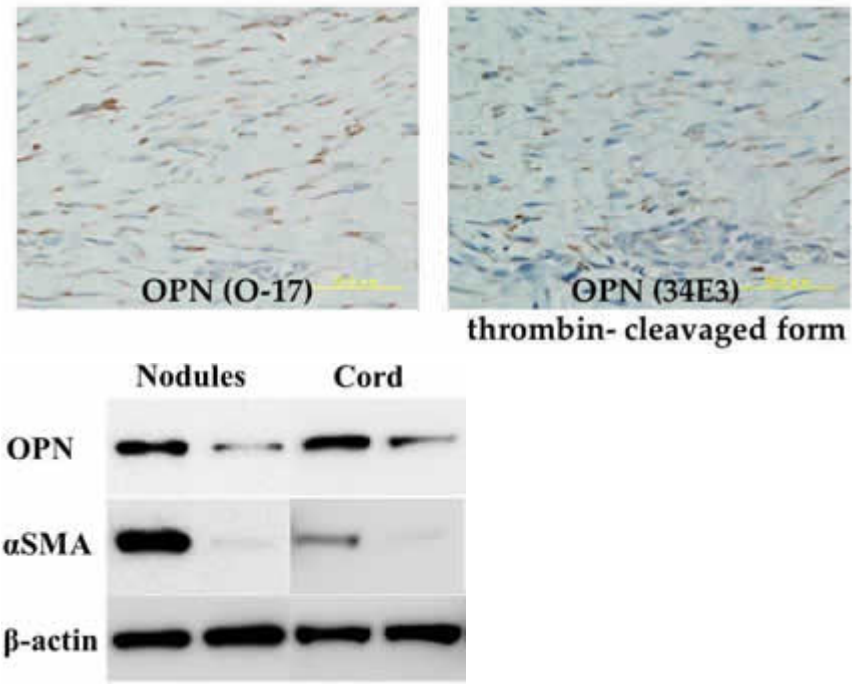
Discussion: The present study showed the expression of OPN in Dupuytren's fascia and was signifincantly co-localized to the expression of αSMA. In other words, the majority of OPN was expressed in myofibroblast in palmer fascia of DC. OPN has been recently recognized as a potential inflammatory cytokine in various inflammatory or fibrotic diseases, which was initially identified in the bone. Lenga et al. described that OPN was required for the differentiation and activity of myofibroblasts formed in response to TGF-β based on in vitro study of OPN-null fibroblasts [3]. Thus, OPN could also involve in the pathologic progression by modulationg the activity of myofibroblast in DC. In addition, there was expression of thrombin-cleaved form in the immunohistochemical analysis of this study. OPN contains several adhesive domain to cells, such as an arginine-glycine-aspartate (RGD)-containing domain that interacts with cell-surface integrins. Cleavage by thrombin can expose the binding sites within OPN, which promote adhesion and migration of inflammatory cells through these alternative sites in an RGD-independent manner. In fact, our previous study showed that thrombin-cleaved OPN was significantly expressed in the synovial fluid of the knee joint in rheumatoid arthritis compared to that in osteoarthris [5]. We suggested that thrombin could play a role in activation of inflammatory cells of the chronic fibrosis thorough the cleavage of OPN in DC. Furthermore, the application of thrombin induced myofibroblast transformation from fibroblasts of the nodules as well as the cord. Nodules must be the main tissue of this pathology because there were high cellularities of fibroblast and myofibroblast as shown

in a number of studies including ours. In addition, this study demonstrated that there were upregulation of αSMA in the cells isolated from the cord after the treatment of thrombin. The fact suggested that thrombin in bleeding during surgery involved in the recurrence of DC, in which high frequency of recurrence was well known with range from 20% to 70%. This severe complication in the surgical treatment could be controlled by the inhibition of thrombin.

Significance: The present study showed that myofibroblast expressed osteopontin (OPN) as well as thrombin-cleaved OPN in the nodules of Dupuytren's contracture (DC). In in vitro study, thrombin induced differentiation into myofibroblast from fibroblasts of both nodules and cords. Thrombin may involve in the pathology of progression and recurrence by direct effect or indirect pathway via cleavage of OPN.

References: [1] Tomasek JJ. Nat Rev Mol Cell Biol 3, 2002; [2] Artuc M. Exp Dermatol 15, 2006; [3] Lenga Y, et al. Circ Res 102, 2008; [4]. Mori R et al. J Exp Med 205, 2008; [5] Hasegawa M et al. J Rheumatol 36, 2009





ORS 2013 Annual Meeting

Poster No: 1154