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Original contribution

The role of TGF-β and myofibroblasts in the arteritis of Kawasaki disease☆

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Summary Inflammation of medium-sized, muscular arteries and coronary artery aneurysms are hallmarks of Kawasaki disease (KD), an acute, self-limited vasculitis of children. We previously reported that genetic variation in transforming growth factor (TGF)-β pathway genes influences both susceptibility to KD and coronary artery aneurysm (CAA) formation. TGF-β signaling has been implicated in the generation of myofibroblasts that influence collagen lattice contraction, antigen presentation, and recruitment of inflammatory cells as well as the generation of regulatory T-cells (Tregs). These processes could be involved in aneurysm formation and recovery in KD. Coronary artery tissues from 8 KD patient autopsies were stained to detect proteins in the TGF-β pathway, to characterize myofibroblasts, and to detect Tregs. Expression of proteins in the TGF-β pathway was noted in infiltrating mononuclear cells and spindle-shaped cells in the thickened intima and adventitia. Coronary arteries from an infant who died on Illness Day 12 showed α-smooth muscle actin (SMA)-positive, smoothelin-negative myofibroblasts in the thickened intima that co-expressed IL-17 and IL-6. CD8+ T-cells expressing HLA-DR+ (marker of activation and proliferation) were detected in the aneurysmal arterial wall. Forkhead box P3 (FOXP3), whose expression is essential for Tregs, was also detected in the nucleus of infiltrating mononuclear cells, suggesting a role for Tregs in recovery from KD arteritis.TGF-β may contribute to aneurysm formation by promoting the generation of myofibroblasts that mediate damage to the arterial wall through recruitment of pro-inflammatory cells. This multi-functional growth factor may also be involved in the induction of Tregs in KD.

1. Introduction

Kawasaki disease (KD) is a self-limited vasculitis that is the most common cause of acquired heart disease in children [1]. Neither the trigger for the acute inflammation nor the mechanisms through which the immune activation is down-regulated are known. Based on echocardiographic data, 20%-30% of KD patients will develop transient dilatation of the coronary arteries and 5%-9% will develop coronary

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artery aneurysms despite timely therapy with intravenous immunoglobulin (IVIG) [2,3].

The importance of transforming growth factor (TGF)-β in KD was initially highlighted by our finding that genetic variation in the TGF-β pathway influences both susceptibility and risk of coronary artery aneurysms (CAA) [4]. Single nucleotide polymorphisms (SNPs) in a ligand (*TGFB2*), a receptor (TGF-β receptor 2: *TGFBR2*), and a key signaling molecule (SMAD family member 3: *SMAD3*) in the pathway were associated with both KD susceptibility and formation of CAA. We also demonstrated that genes in the TGF-β signaling pathway were differentially expressed in whole blood during the acute and convalescent stages of KD. Based on these data, we predicted that TGF-β signaling would be important in the genesis of vasculitis. TGF-β signaling has been implicated in the generation of myofibroblasts that influence collagen lattice contraction, antigen presentation, and recruitment of inflammatory cells as well as the generation of regulatory T-cells (Tregs). Therefore we postulated that TGF-β induces transformation of cells of different lineages to myofibroblasts that mediate damage to the arterial wall. We further postulated a role for TGF-β in the genesis of Tregs and the down-regulation of inflammation based on our T-cell cloning studies using acute KD peripheral blood [5].

In this study, we investigated expression of proteins in the TGF-β pathway in the coronary arterial wall from KD autopsies. To test the hypothesis that myofibroblasts in the arterial wall participate in aneurysm formation, we performed immunohistochemical (IHC) staining for markers of smooth muscle cells (SMCs) (smoothelin), myofibroblasts (α-smooth muscle actin [SMA]), pro-inflammatory cytokines (Interleukin [IL]-17 and IL-6), mature T-cells (CD3+), cytotoxic T-cells (CD8+) and their activation/proliferation marker human leukocyte antigen (HLA)-DR.The potential anti-inflammatory effect of TGF-β was tested by IHC staining for nuclear FOXP3, a marker for Tregs.

2. Materials and methods

2.1. Subjects

All subjects met American Heart Association (AHA) clinical criteria for KD during their acute illness [6]. CAA were defined according to Japanese Ministry of Health criteria as a coronary artery segment with an internal diameter 1.5 times that diameter of the adjacent segment [7]. All protocols were approved by the Institutional Review Board at University California, San Diego (UCSD) and Toho University, Japan, and written parental consent was obtained for the use of all tissues.

2.2. Tissues

We obtained formalin-fixed, paraffin-embedded tissues from 8 KD patients (age 2 mo - 5 y 2 mo) who expired 12 days-20 m after onset of fever and from 2 age-similar control children who died from complications of Tetralogy of Fallot (TOF) and idiopathic acute encephalopathy.

2.3. Immunohistochemical staining

Tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. Antigen retrieval was performed either in citrate buffer (unmasking solution, Vector Laboratories, Cat. H-3300) or in ethylenediaminetetraacetic acid (EDTA) (pH 9.0 for CD8) buffer in a microwave oven for 10 minutes. Slides were incubated with 1.5% normal goat or horse serum blocking solution at room temperature for 30 min (Vector Laboratories) then incubated in PBS with 1.5% goat or horse serum overnight at 4°C or for 60 minutes at room temperature with the following antibodies: TGF-β2 (sc-90) , TGF-βR2 (sc-400), pSMAD2/3 (sc-11769), smoothelin (sc-28562), IL-17 (sc-7927) from Santa Cruz Biotechnology; Connective tissue growth factor (CTGF) (ab5097), IL-6 (ab6672), Forkhead box P3 (FOXP3) (ab10901), HLA-DR (ab92511) from Abcam; α-SMA (M0851), CD3 (N1580), CD8 (IS623) from Dako (detailed information in Supplemental Table 1). Antibodies were detected using biotin-avidin, ABC (Vectastain Rabbit: PK-6101, Mouse: PK-6102) followed by visualization with the AEC substrate kit (Invitrogen 00–2007) or biotin-streptavidin, LSAB2 System-HRP (DAKO K0675) followed by coloring with AEC or PowerVisionPoly-AP IHC Detection Systems (Leica Biosystems, Cat. PV6133) and coloring with permanent red (Dako, K0640) according to the manufacturer's instructions (Supplementary Table 1). Negative staining controls were performed using either normal rabbit immunoglobulin G (IgG) (Dako, Cat. X0936) or mouse universal negative control (Dako, Cat. N1698).

For immunofluorescent double-staining, tissue sections were treated as described previously except without

Fig. 1 TGF-β2, TGF-βR2, pSMAD3 and CTGF expression in coronary arterial wall in KD and control autopsy tissues. Case 1, 2, 3, 6 (KD), and 9 (Tetrology of Fallot [TOF] negative control) (x100): H&E staining of coronary arteries. Boxed area indicates location of section used for IHC shown in A-L. TGF-β2 (A-C): Positive staining of mononuclear and spindle-shaped cells in KD arteries (A and B). Control shows rare positively stained spindle-shaped cell in adventitia (C). TGF-βR2 (D-F): Positive staining of spindle-shaped cells (arrowhead) only in KD arteries (D and E) but not control tissues (F). pSMAD3 (G-I): Positive nuclear staining of spindle-shaped cells (arrowhead) in adventitia and mononuclear cells (arrow) in intima from KD artery (G and H) but not control (I). CTGF (J-L): Positive staining of spindle-shaped cells (arrowhead) in adventitia and intima of KD artery (J and K) and media of control artery (L). TGF-β2: transforming growth factor-β2, TGF-βR2: TGF-β receptor 2, pSMAD3: phosphorylated SMAD3, CTGF: connective tissue growth factor.