Role of the PTEN/PI3K/VEGF pathway in the development of Kawasaki disease

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Abstract. Kawasaki disease (KD) is a disease of unknown etiology and the leading cause of childhood acquired heart disease. In this study, the significance of the phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K)/vascular endothelial growth factor (VEGF) pathway in the development of KD was investigated in a rabbit model. Rabbits were divided into the control group, which received saline injection, and the experimental group, which was treated with bovine serum albumin to induce arthritis and KD. After 1, 7 and 30 days the animals were sacrificed, and the white blood cell count, serum VEGF, and serum creatine kinase (CK) levels were measured. The coronary artery was examined histologically as well as immunohistochemically for PTEN and PI3K. After the induction of arthritis, coronary artery of the rabbits showed endothelial cell swelling, osteoporosis, necrosis and inflammatory cell infiltration. PTEN expression in these rabbits increased with the increasing number of modeling days. The expression of PI3K showed a decreasing trend. The number of white blood cells in rabbits after KD modeling were significantly higher than those in the controls. One day and 7 days after modeling the serum VEGF level in KD rabbits was significantly higher than that in the control group after 1 and 7 days followed by a decrease by 30 days. There was no significant change in serum CK on the day after the modeling, and the serum CK level was significantly higher after 7 and 30 days. In conclusion, the expression of PTEN/PI3K was altered at different stages of KD. PTEN expression gradually increased with the disease progression, while the expression of PI3K gradually decreased. Serum markers indicated that the PTEN/PI3K/VEGF signaling pathway is important in the vascular injury in KD.

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Introduction

Kawasaki disease (KD) is a disease of unknown etiology, identified primarily in children with systemic vasculitis syndrome, that has become the leading cause of childhood acquired heart disease (1). The main pathological change of KD is systemic non-specific vasculitis, possibly with severe cardiovascular complications. Coronary arthritis can cause coronary artery dilatation, and coronary artery stenosis, leading to myocardial ischemia and when severe leading to myocardial infarction (2-5). KD pathogenesis is not clear, and is associated with infection factors and superantigen-mediated immune responses and the genetic susceptibility of children (6). Previous findings revealed that factors such as nuclear factor-κB, nitric oxide, vascular endothelial growth factor (VEGF), and matrix metalloproteinases are involved in the occurrence and development of KD (7-10).

In the present study, intravenous bovine serum albumin was used to replicate the experimental rabbit model of vasculitis. VEGF was measured in the early, middle and recovery stages of modeling. At the same time, the phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K) signaling pathway that regulates VEGF, was examined to determine the role of the PTEN/PI3K/VEGF pathway in KD at different stages.

Materials and methods

Materials

Experimental animals. Twelve rabbits of 9-12 weeks old, were provided by the Experimental Animal Department of China Medical University. The rabbits were kept in an environment with a temperature of 26˚C and humidity of 70%. The animals were provided with food and water *ad libitum* during the rearing period. Permission was obtained from the Institutional Ethics Committee to conduct the animal experiment.

Instruments. The Thermo Scientific Heraeus Biofuge Stratos Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA); RM2235 paraffin section machine (Leica, Mannheim, Germany); DH101 electric heating constant temperature air blast drying box (Beijing Lee Kang Science and Technology Development Co., Ltd., Beijing, China); BX43 microscope (Olympus Corporation, Tokyo, Japan); and MSHOT MC50

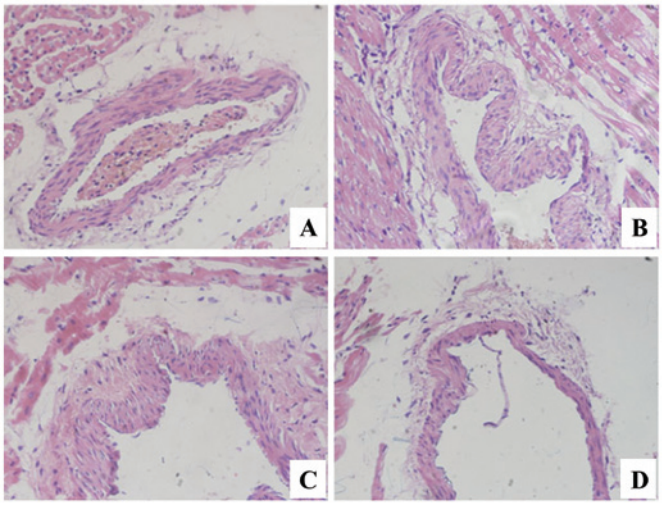

Figure 1. Pathological tissue of hematoxylin and eosin staining in 1, 7 and 30 days after modeling the rabbit with Kawasaki disease: (A) control group; (B) 1 day after modeling; (C) 7 days after modeling; and (D) 30 days after modeling.

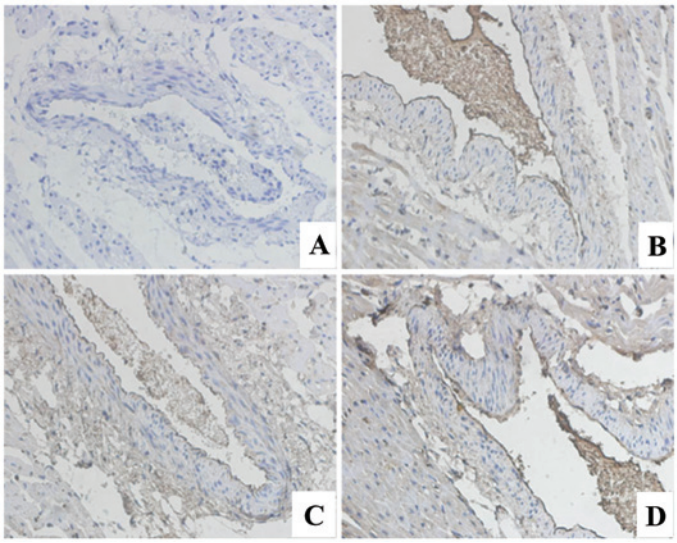

Figure 2. Phosphatase and tensin homolog immunohistochemical pattern in 1, 7 and 30 days after modeling the rabbit with Kawasaki disease: (A) control group; (B) 1 day after modeling; (C) 7 days after modeling; and (D) 30 days after modeling.

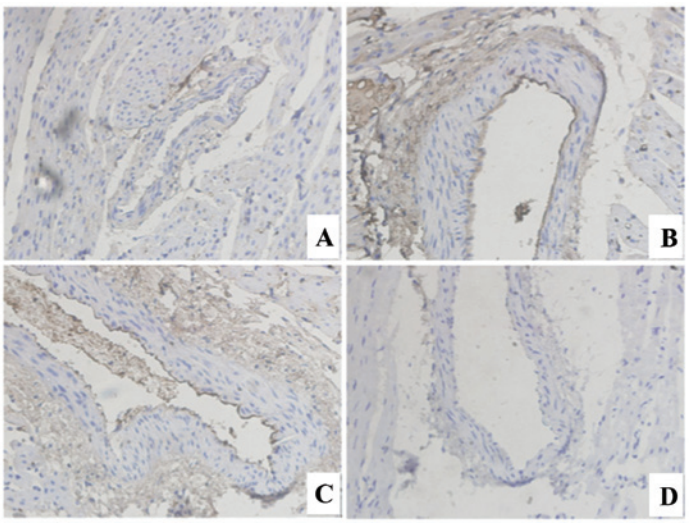

Figure 3. Phosphoinositide 3-kinase immunohistochemical pattern in 1, 7 and 30 days after modeling the rabbit with Kawasaki disease: (A) control group; (B) 1 day after modeling; (C) 7 days after modeling; and (D) 30 days after modeling.

Table I. Effects of the number of white blood cells of the rabbit model with Kawasaki disease (KD) in 1, 7 and 30 days after modeling.

White blood cell count (10^9/l)	Group 1 day (n=2)	Group 7 days (n=2)	Group 30 days (n=2)
Control group	5.9±1.2	5.7±1.3	6.0±1.3
Group KD	14.6±2.3[a]	21.4±2.2[a]	11.1±1.9[a]

Compared with the corresponding control group, [a]P<0.01.

Table II. Effects of serum VEGF of rabbit model with Kawasaki disease (KD) in 1, 7 and 30 days after modeling.

VEGF (ng/l)	Group 1 day (n=2)	Group 7 days (n=2)	Group 30 days (n=2)
Control group	33.9±6.7	35.9±7.3	34.5±5.9
Group KD	89.1±15.5[a]	76.9±9.9[a]	19.8±4.4[a]

Compared with the corresponding control group, [a]P<0.01. VEGF, vascular endothelial growth factor.

Table III. The effect of serum CK of the rabbit model with Kawasaki disease (KD) in 1, 7 and 30 days after modeling.

CK (U/l)	Group 1 day (n=2)	Group 7 days (n=2)	Group 30 days (n=2)
Control group	635.7±169.3	640.5±174.7	629.4±163.8
Group KD	637.6±127.4	1441.9±637.3[a]	1165.68±256.4[a]

Compared with the corresponding control group, [a]P<0.01. CK, creatine kinase.

micro imaging system (Guangzhou Ming-Mei Technology Co., Ltd., Guangzhou, China), were used in the present study.

Reagents. The ELISA kit for VEGF, PTEN and PI3 rabbit anti-mouse polyclonal antibodies were purchased from the Beijing Boosen Biological Technology Co., Ltd. (cat: bs-0686R, bs-0128R, Beijing, China). The General Type goat anti-rabbit monoclonal secondary antibody and DAB color agent, were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (cat: PV-6001, Beijing, China). The creatine kinase (CK) kit was purchased from Beijing Boding Biological Engineering Co., Ltd. (Beijing, China).

Methods

KD rabbit model. Twelve of 9 to 12-week-old rabbits were divided into the experimental group (n=6) and control group (n=6).

Experimental group. The 6 rabbits in the experimental group, were received 2.5 mg bovine serum/kg (in 2.5 ml volume/kg) as intravenous injection (9). After 2 weeks, the experimental group was given another dose of 2.5 mg bovine serum/kg intravenous slow bolus to induce arthritis, forming vasculitis.

Control group. The 6 rabbits of the control group, were intravenously injected with 2.5 ml/kg saline. Two weeks later, 2.5 ml/kg normal saline was intravenously injected.

Tissue pathology examination

Paraffin section hematoxylin and eosin (H&E) staining. The two groups of rabbits, respectively 1, 7 and 30 days after modeling were sacrificed (n=2, at each time point). The chest was opened to expose the heart, and the coronary artery was isolated and immediately fixed in neutral-buffered formalin. The fixed arteries were embedded in paraffin block, sectioned and stained with H&E. Histological changes were observed under a light microscope (CX31, Olympus, Tokyo,Japan).

PTEN and PI3K immunohistochemical staining of the paraffin sections. The paraffin sections were extracted from the coronary artery of the rabbits. Immunohistochemical staining procedures were carried out as follows: the slides were kept at 65˚C in an oven for 6 h, dewaxed in dimethylbenzene, dehydrated with gradient ethanol and rinsed with double-distilled water to block endogenous catalase. Following treatment with the primary and secondary antibodies at 4˚C, DAB was used to develop color and observed under the microscope. Images were captured and analyzed using IPP software (Image-Pro Plus) 6.0. (Media Cybernetics, Baltimore, MD, USA).

Hematology test. Blood was collected for the white blood cell count at 1, 7 and 30 days after modeling. The serum CK and VEGF levels were measured according to the manufacturer's instructions.

Results

Immunohistochemical staining for PTEN, PI3K and coronary H&E staining. H&E staining (Fig. 1) showed the changes of endothelial cell swelling, osteoporosis, necrosis, and inflammatory cell infiltration in the coronary artery tissue of the experimental group, which was consistent with the pathological characteristics of KD, suggesting that the model was successful.

Immunohistochemical staining for PTEN and PI3K. The expression of PTEN in the model group was significantly higher than that of the control group. PTEN expression of increased gradually with the increase in the number of days after modeling (Fig. 2). The expression of PI3K showed the opposite trend. Compared with the normal rabbit, the expression of PI3K in the coronary artery of rabbits was lower. The expression of PI3K showed a gradually decreasing trend following 1, 7 and 30 days of modeling (Fig. 3).

Changes in the blood parameters of rabbits in the model group and control groups

Whole white blood cell count. The number of white blood cells of rabbits in the model group was significantly increased

compared to that of the control group (Table I), which is consistent with the hematological manifestation of arthritis. At 7 days after modeling, the number of white blood cells increased compared to 1 day after modeling. At 30 days after modeling, the number of white blood cells decreased, although their number was higher than that of the control group.

Serum VEGF results. Serum VEGF levels in rabbits on the day of modeling increased significantly compared with the control group (Table II). At 1 week after modeling the serum level of VEGF initially increase but was then decreased. However, this serum level was higher than that of the control group. At 30 days after modeling, the VEGF levels were significantly decreased, and lower than those of the control group.

Serum CK results. On the day of modeling, serum CK exhibited no obvious change (Table III). However, 1 week to 1 month after modeling, serum CK increased significantly compared with that of the control group.

Discussion

The pathological changes of KD mainly show as a systemic non-specific vasculitis, which mainly involves small and medium-sized arteries, especially coronary arteries (2-5). The present study produced a KD model as previously described, using bovine serum albumin to immunize rabbits (11). The pathological changes of the coronary artery were similar to the vascular lesions of KD in this model. At 1, 7 and 30 days after injection of bovine serum albumin, the wall of the coronary artery gradually became thinner, deformed and enlarged. Serum white blood cells increased gradually and the amount of WBC reached their maximum after 21 days. The CK level was the highest on day 21, which indicated segment cardiac damage at different time points of the model.

VEGF is mainly generated by vascular smooth muscle cells and released during vascular inflammation. The latter process can induce fractures, collagenase and metalloprotease synthesis, accelerate small veins and capillary cracks, express endothelial cell adhesion of molecules expression and cause peripheral vascular edema, during the pathogenesis of KD (12). Previous findings have shown that serum VEGF level in children with KD was significantly higher than that in remission and normal children (13). The current findings show that serum VEGF significantly increased on days 1 and 7, but on day 30 the level of VEGF was decreased. This result may be due to the fact that the animal model and Kawasaki patients are different, due to the sampling time.

In order to determine the causes of VEGF change, we detected changes in the PTEN/PI3K pathway. The expression of PI3K and VEGF with active mutation in tumor cells is associated with an increased expression of angiogenesis (14-16). The overexpression of PI3K and AKT also induces VEGF transcription and promotes the formation of new blood vessels.

PI3K can produce PIP3, phosphorylate the Ser473, Thr308 site of AKT, activate AKT, participate in the transcription and translation of intracellular-associated genes and promote the development of normal blood vessels. Previous studies have found that LY294002, a PI3K inhibitor, has a role of anti-angiogenesis in quality microenvironment and retinopathy of tumor tissue (17,18), while PTEN is a phosphatase, which is a major negative regulator of PIP3, by dephosphorylating and thus antagonizing the PI3K/AKT pathway. If PTEN is inactivated, PI3K/AKT sustains activation, resulting in cell division, increased volume, apoptosis and tumor angiogenesis (19-21), suggesting that the PTEN/PI3K/VEGF signaling pathway plays an important role in vascular injury.

In conclucion, immunohistochemistry was used in the current study to show that in the rabbit model, the expression of PTEN/PI3K was different at different stages. In addition, the expression of PTEN gradually increased, whereas the expression of PI3K was gradually reduced. There was a negative correlation between PTEN and PI3K. Changes in the serum VEGF level suggest that the PTEN/PI3K/VEGF signaling pathway plays an important role in the development of KD.

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