Pediatr Cardiol (2011) 32:455–460
DOI 10.1007/s00246-011-9900-4

ORIGINAL ARTICLE

The Functions of Endothelial Progenitor Cells Were Significantly Improved After Treatment With Intravenous Immunoglobulin and Aspirin in Children With Kawasaki Disease

Ming Guo Xu · Li Na Men · Ying Zu · Chun Yu Zhao · Xiang Chun Meng · Tao Wang

Received: 23 July 2010 / Accepted: 31 January 2011 / Published online: 16 February 2011

Abstract We sought to determine the effects of treatment with intravenous immunoglobulin (IVIG) and aspirin on the functions of endothelial progenitor cells (EPCs) in patients with Kawasaki disease (KD) as well as its relationship with concentrations of tumor necrosis factor-α (TNF-α) and high-sensitivity C-reactive protein (hs-CRP). Ten KD patients in the acute phase of their disease were recruited. We investigated EPC functions in children with KD before and after treatment with IVIG and aspirin. In vitro assays were used to measure the functions, including proliferation, adhesion, and migration activities, of EPCs. Plasma levels of TNF-α and hs-CRP were also assessed. All of the data were assessed before and at 7 days after treatment initiation. EPC functions after 7 days of treatment with IVIG and aspirin were significantly improved than they were before treatment with IVIG and aspirin. Treatment with IVIG and aspirin significantly decreased TNF-α and hs-CRP concentrations. There was a significant linear regression relationship between decreased plasma TNF-α levels, hs-CRP levels, and increased functions of circulating EPCs. The results of our study indicate that the functions of circulating EPCs improved after treatment with IVIG and aspirin, which may be related to decreased concentrations of TNF-α and hs-CRP.

Keywords Kawasaki disease · Intravenous immunoglobulin · Endothelial progenitor cell

M. G. Xu (✉) · C. Y. Zhao · X. C. Meng · T. Wang
The Cardiovascular Center, Shen-Zhen Children's Hospital, No. 7019, Yi Tian Rd, Fu Tian District, Shen-Zhen 518026, China
e-mail: nihaomingguo@yahoo.com.cn

L. N. Men
The Neurology Department, Shen-Zhen Children's Hospital, No. 7019, Yi Tian Rd, Fu Tian District, Shen-Zhen 518026, China

Y. Zu
The Pediatric Research Institute, Shen-Zhen Children's Hospital, No. 7019, Yi Tian Rd, Fu Tian District, Shen-Zhen 518026, China

Introduction

Kawasaki disease (KD) is a systemic vasculitis disease that mainly damages moderate- and small-sized blood vessels, especially in coronary arteries [4, 14, 15]. Until now, the exact mechanisms of coronary artery injury have not been completely clear. However, injury of the coronary artery in patients with KD may be attributed to the immune system disorder, the excess secretion of mediators of inflammation and cytokines, and the insufficiency of vascular repair [5, 10, 16, 17, 30]. Endothelial progenitor cells (EPCs) are clusters of stem cells that can differentiate into competent and mature endothelial cells [2]. Data have shown that EPCs represent the reparation factor of the vascular wall and are closely related to cardiovascular well being [3, 12, 28].

Our previous data showed that EPC functions in acute-phase KD patients were significantly impaired, perhaps delaying the reparation process of coronary arteries [30]. Intravenous immunoglobulin (IVIG), together with aspirin, is a world-wide accepted method to treat KD and can effectively prevent injury of the coronary arteries [25]. However, there has been no report about the effects of treatment with IVIG and aspirin on EPC function in KD patients. It has been proven that concentrations of tumor necrosis factor-α (TNF-α) and high-sensitivity C-reactive protein (hs-CRP) are significantly increased in acute-phase KD patients [1, 19, 23]. The results of several studies have

indicated that high concentrations of TNF-α and hs-CRP may impair EPC structure and function [7, 8, 31]. Therefore, the present study was designed to investigate changes in EPC function before and after treatment with IVIG and aspirin as well as its relationship with concentrations of TNF-α and hs-CRP. To our knowledge, our study is the first to propose that treatment with IVIG and aspirin may decrease coronary artery injury by improving EPC function.

Materials and Methods

Patients and Blood Collection

We studied 10 patients with KD (age 5 months to 3 years [median 20 months]; male-to-female ratio of 7:3). Informed consent was obtained from the parents of all patients. All patients were hospitalized at our hospital from July 2008 to March 2010. The patients were enrolled within 7 days of the onset of illness, with day 1 being defined as the first day of fever. All patients fulfilled the diagnostic criteria for KD as established by the Japanese Kawasaki Disease Research Committee and were considered typical cases. The initial course of treatment with IVIG and aspirin was started on days 5 to 7. All patients were scheduled to receive 2g/kg IVIG for 1 day. Aspirin (50 mg/kg/day) was also administered during the acute phase and was gradually decreased to 5 mg/kg/day within 2 weeks after fever ceased. Blood samples were collected before and at 7 days after treatment initiation. The study protocol was approved by the Ethics Committee of our hospital.

Cell Culture and EPC Characterization

The procedure was performed as in our previous study [29, 30]. Peripheral blood mononuclear cells were isolated using Ficoll density gradient centrifugation and suspended in a 25-cm^2 cell culture bottle coated with fibronectin (Hematological Technologies) using endothelial cell basal medium-2 (Clonetics, San Diego, CA) supplemented with EGM-2 MV (Clonetics) and incubated at 37°C in a humidified environment with 5% carbon dioxide (CO_2). The culture medium was replaced every 4 days. To determine the EPC phenotype, the attached induced cells were incubated with 1,10-dioctadecyl-3,3,30,30-tetramethylindo-carbocyanine perchlorate–labeled acetylated LDL (Dil-acLDL; 10 μg/ml; Molecular Probes) at 37°C for 1 h. Then the cells were fixed with 4% paraformaldehyde for 30 minutes at 37°C and incubated with FITC-labeled Ulex europeus agglutinin (FITC-lectin, 10 μg/ml; Sigma) for 4 h at 37°C. After being stained, the cells were observed with a phase-contrast fluorescent microscope (Olympus). Cells showing double-positive fluorescence of Dil-acLDL and FITC-lectin were identified as differentiating EPCs.

EPC Function Assay: Proliferation Adhesion and Migration Activity

The following procedure was also performed as in our previous study [29, 30]. EPCs were digested with 0.25% trypsin and then cultured in serum-free medium in a 96-well culture plate (200 μl/well). After being cultured for 24 h, EPCs were supplemented with 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 g/l; Fluka) and incubated for another 4 h. The supernatant was discarded by aspiration, and the EPC preparation was shaken with 200 μl dimethylsulfoxide for 10 minutes before optical density was measured at 490 nm.

EPCs (2.5×10^4/well) were seeded onto 24-well plates precoated with fibronectin and incubated for 30 minutes at 37°C with 5% CO_2. After washing three times with phosphate-buffered saline (PBS), attached cells were counted. Adhesion activity was evaluated as the mean number of attached cells in 5 high power fields (200×)/well.

EPC migrations were evaluated with a modified Boyden chamber method. EPC suspension (2.5×10^4 cells/well) was placed in the upper chamber with endothelial basal medium (EBM), and the chamber was placed in a 24-well culture dish containing EBM and 50 ng/ml VEGF (Hematological Technologies, Essex Junction). The chamber was incubated for 24 h, and the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with 4′,6-diamidino-2-phenylindole. Cells migrating into the lower chamber were counted manually in three random microscopic fields.

Plasma TNF-α and hs-CRP Detection

Blood samples were separated at 4°C and stored at –20°C. hs-CRP was measured at 550 nm with the use of the Particle-Enhanced Immunoturbidimetric Assay (Orion Diagnostica) and performed by a specialist who was blinded to the study assignment.

Plasma levels of TNF-α were measured by enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer's instructions. Results were compared with standard curves. Measurements were performed in duplicate.

Statistical Analysis

Results are presented as means ± SDs and were analyzed with SPSS software (version 13.0). Statistical significance was evaluated by paired Student *t* test. Bivariate

correlations were calculated according to Pearson, and $P < 0.05$ was considered significant.

Results

EPC Function Before and After Treatment with IVIG and Aspirin

Figure 1 shows that the in vitro-cultured EPCs were characterized by double staining with Dil-acLDL and FITC-lectin. Figure 2 shows the change in EPC function. The EPC migratory response increased significantly after treatment with IVIG and aspirin (5.50 ± 1.80 vs. 7.90 ± 2.23 cells/high power field [$P < 0.01$]). Similarly, the proliferative and adhesive activities also improved (0.38 ± 0.08 vs. 0.50 ± 0.08 [$P < 0.01$] and 6.50 ± 1.65 vs. 10.7 ± 1.89 cells/high power field [$P < 0.05$]).

Plasma TNF-α and hs-CRP Levels Before and After Treatment With IVIG and Aspirin

Figure 3 shows that TNF-α and hs-CRP levels (48.40 ± 7.42 vs. 32.00 ± 6.41 pg/l [$P < 0.05$] and 84.10 ± 24.26 vs. 13.40 ± 3.03 mg/l [$P < 0.05$]) decreased significantly after treatment with IVIG and aspirin.

Association Between EPC Functions and Plasma TNF-α and hs-CRP Levels

There was a significantly linear regression relationship between decreased plasma TNF-α levels and increased EPC functions (Fig. 4). Similarly, decreased plasma hs-CRP concentration was also related with increased EPC functions (Fig. 5).

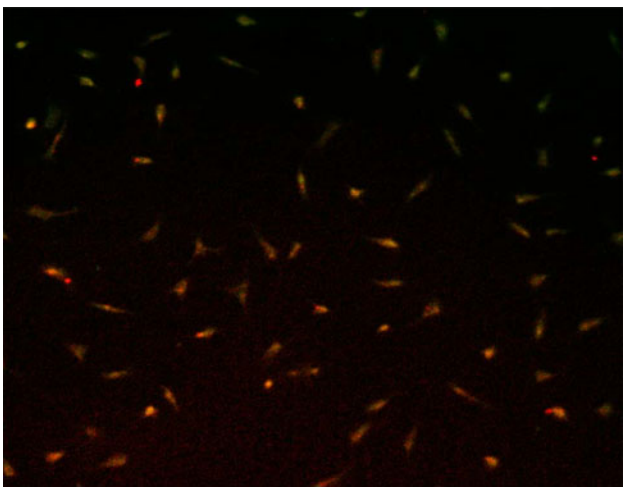

Fig. 1 Identification of EPCs stained by Dil-acLDL and FITC-Lectin. Magnification ×100

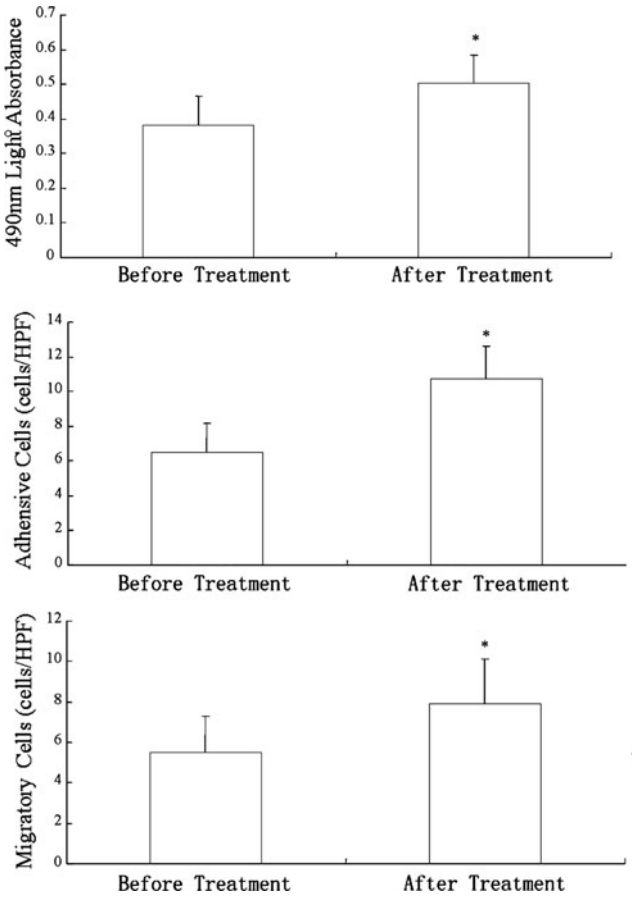

Fig. 2 The proliferative, adhesive, and migratory activities of cultured EPCs before and after 7 days of treatment with IVIG and aspirin. Data are given as means ± SDs. *$P < 0.05$ vs. before IVIG and aspirin treatment. *HPF* high power field

Discussion

The present study investigated the functions of in vitro-cultured EPCs collected from KD patients before and after treatment with IVIG and aspirin. Our major findings were that EPC functions significantly improved after 7 days of treatment with IVIG and aspirin in KD patients and that concentrations of TNF-α and hs-CRP were statistically decreased after 7 days of treatment with IVIG and aspirin compared levels before treatment onset. Meanwhile, we found that improved EPC functions related positively to decreased plasma TNF-α and hs-CRP levels in our study. The present study indicates that treatment with IVIG and aspirin may effectively enhance EPC functions and that the mechanisms underlying the process may be related to decreased plasma TNF-α and hs-CRP levels. Enhanced EPC function may accelerate the repair of vascular injury, which probably promotes a positive prognosis for KD patients.

Coronary artery injury associated with KD may be the major cause of acquired heart disease in children [6].

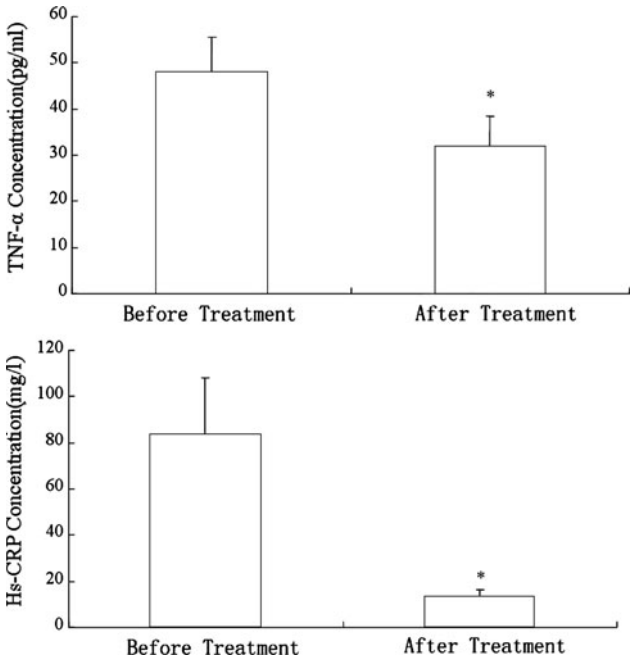

Fig. 3 Plasma TNF-α and hs-CRP concentrations before and after 7 days treatment with IVIG and aspirin. Data are given as means SDs. $*P < 0.05$ vs. before treatment with IVIG and aspirin

Furthermore, coronary artery aneurysm (CAA) may be the most serious complications in KD patients and may lead to death [6]. Some of our KD patients presented with ischemic heart disease because of coronary artery stenosis or calcification. Persistent CAA correlates with impaired functions of endothelial cells and smooth muscle cells of coronary arteries [9, 24]. Even if the CAA disappears, the morphology of the vascular wall and the functions of vascular cells remain to be abnormal. Data have shown that adult patients with a history of KD had systemic vascular endothelial dysfunction and that a history of KD could possibly be one of the risk factors for early onset of atherosclerosis [9, 22]. Therefore, preventing and healing coronary artery injury is important in KD patients.

Data have shown that EPCs play a pivotal role in maintaining a normal state of the vascular wall [2, 3, 11, 12]. In humans, the numbers and functions of EPCs are positively associated with endothelial function [12]. In animal research, it has been proven that enhanced levels of peripheral circulating EPCs were attributed to improved rate of re-endothelialization and inhibition of neointimal formation in arteries denuded by balloon injury [11, 27]. Moreover, increased EPC levels were associated with a decreased risk of death from cardiovascular disease [28]. The previously mentioned data indicated that the level of peripheral circulating EPCs in patients with heart disease may affect cardiovascular outcomes in the future. We postulated that the circulating level of EPCs in KD patients may also influence future cardiovascular health.

In the present study, we found that the functions of circulating EPCs increased significantly in KD patients after treatment with IVIG and aspirin. To investigate the possible mechanism of this phenomenon, we measured levels of plasma TNF-α and hs-CRP before and after treatment with IVIG and aspirin. Data have shown that TNF-α has negative effects on EPC function [31]. It has also been reported that plasma hs-CRP is also negatively associated with circulating EPC functions [7, 8]. We found that the plasma concentrations of TNF-α and hs-CRP decreased in KD patients after treatment with IVIG and aspirin. Furthermore, a positive correlation was found between enhanced EPC functions and decreased TNF-α and hs-CRP concentrations in KD patients, which may indicate that increased TNF-α and hs-CRP levels result in impaired EPC functions in acute-phase of KD.

Several studies have shown that TNF-α may accelerate coronary arterial injury through recruiting leukocyte

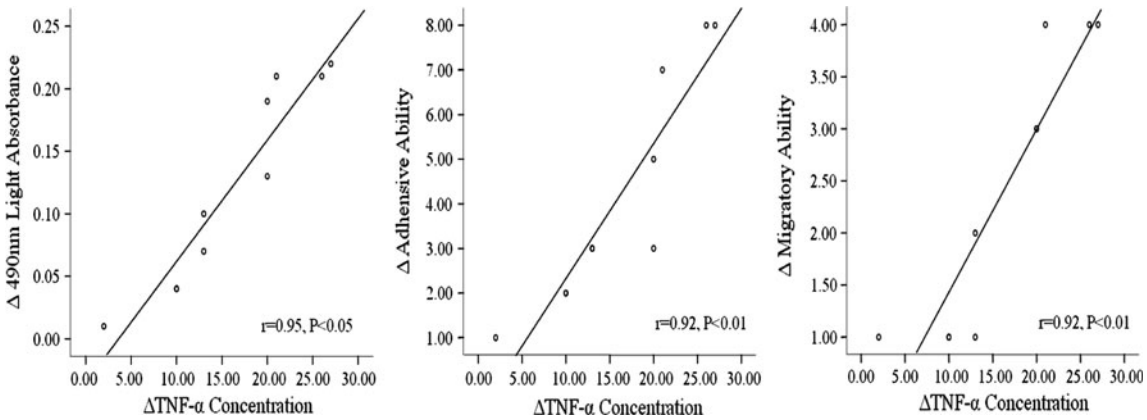

Fig. 4 Linear regression relationship between decreased plasma TNF-α level and promoted functions of circulating EPCs in KD patient group. Δ = absolute value of the change before and after IVIG and aspirin treatment

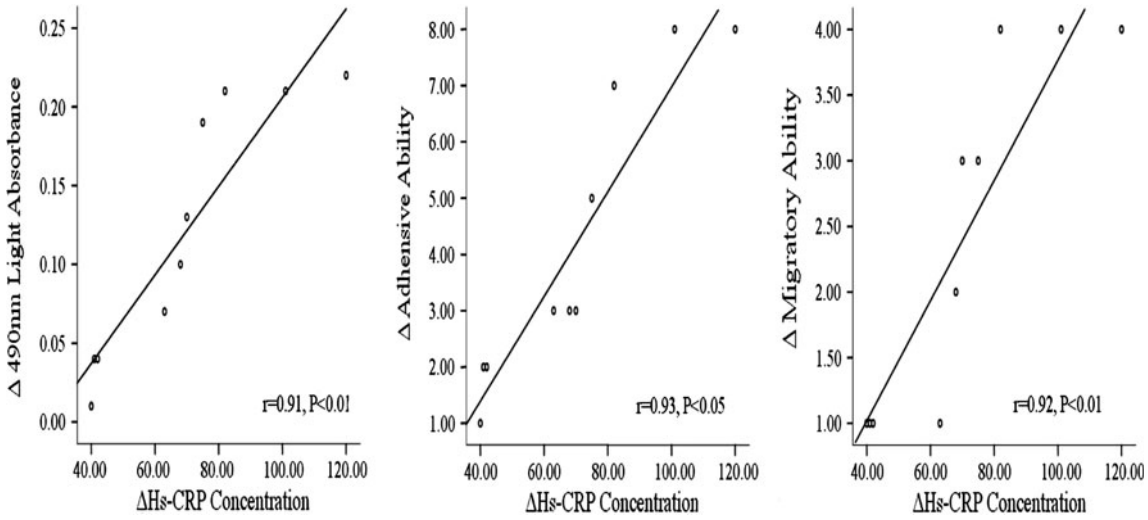

Fig. 5 Linear regression relationship between decreased plasma NO level and promoted functions of circulating EPCs in KD patient group. Δ = absolute value of the change before and after IVIG and aspirin treatment

aggregation, elastin degradation, and other inflammatory injury [13, 18]. However, previous studies have focused on the direct injury processes mediated by TNF-α in KD patients. In the present study we are the first to suggest that TNF-α may also accelerate the damage of coronary arteries through impairing EPC functions, which are regarded as reparative factors for maintaining vascular integrity. Therefore, blocking TNF-α production and its downstream reactions will effectively interrupt the injury process mediated by TNF-α and may accelerate the recovery of coronary arterial injury through improved EPC functions. In vitro study simulating the pathogenesis of KD showed that IVIG treatment significantly inhibited the production of TNF-α [17]. We postulated that improved EPC functions by decreasing TNF-α production may be attributed to the beneficial effects of treatment with IVIG and aspirin in KD patients.

hs-CRP has been widely used as a marker of indicating inflammation reaction and tissue injury; nevertheless, hs-CRP is a nonspecific marker. Its concentration increases in several conditions, such as acute inflammation, surgical trauma, microorganism infection, and autoimmune disease [20]. Further studies have proven that hs-CRP can also directly injure cells, including endothelial cells and endothelial progenitor cells [7, 8, 21]. Decreased hs-CRP concentration in KD patients after treatment with IVIG and aspirin may inhibit direct injury to cells and the vascular wall, thereby protecting the reparative ability of EPCs. Therefore, we consider that treatment with IVIG and aspirin has two advantages in KD patients: to suppress direct inflammatory injury and to recover reparative functions, such as that of EPCs.

Limitations

It should be noted that the present study had the following limitations. First, we proposed that decreased TNF-α and hs-CRP concentrations contributed to improved EPC functions after IVIG infusion. The definitive cause-and-effect relation between them, however, requires further studies. Second, our study cannot prove that enhanced EPC functions directly participate in the reparative process of coronary arteries after IVIG infusion or that such treatment has long-term effects on the cardiovascular system in KD patients. Third, several other agents, such as nitric oxide (NO) and vascular endothelial growth factor (VEGF), play a pivotal role in modulating EPC numbers and functions. In the present study, we did not determine whether IVIG infusion can change the production of NO and VEGF in KD patients or its potential relationship with EPC function.

In conclusion, our study is the first to show that EPC functions improved in KD patients after treatment with IVIG and aspirin, which may be related to the ensuing decreased TNF-alpha and hs-CRP concentrations. Enhanced EPC levels perhaps contributed to the beneficial effects of treatment with IVIG and aspirin in KD patients. Accordingly, we postulate that some drugs, such as statins and berberine [26, 29], which can mobilize EPCs from bone marrow and enhance the functions of circulating EPCs, will improve the prognosis for KD patients, especially those cases that are complicated with coronary arterial injury.

Acknowledgments This work was financially supported by the Guang Dong Province Medical Science Foundation (Grant No.

B2009226). We thank the nurses who work in our center (The Cardiovascular Center, Shen-Zhen Children's Hospital, China) for their assistance in collecting blood sample from volunteers.

References

1. Ahn SY, Jang GC, Shin JS, Shin KM, Kim DS (2003) Tumor necrosis factor-alpha levels and promoter polymorphism in patients with Kawasaki disease in Korea. Yonsei Med J 44(6): 1021–1026
2. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T et al (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275(5302):964–967
3. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H et al (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. Embo J 18(14):3964–3972
4. Brogan PA, Bose A, Burgner D, Shingadia D, Tulloh R, Michie C et al (2002) Kawasaki disease: an evidence based approach to diagnosis, treatment, and proposals for future research. Arch Dis Child 86(4):286–290
5. Brogan PA, Shah V, Klein N, Dillon MJ (2004) Vbeta-restricted T cell adherence to endothelial cells: a mechanism for superantigen-dependent vascular injury. Arthritis Rheum 50(2):589–597
6. Burns JC, Glode MP (2004) Kawasaki syndrome. Lancet 364(9433):533–544
7. Chen J, Huang L, Song M, Yu S, Gao P, Jing J (2009) C-reactive protein upregulates receptor for advanced glycation end products expression and alters antioxidant defenses in rat endothelial progenitor cells. J Cardiovasc Pharmacol 53(5):359–367
8. Fujii H, Li SH, Szmitko PE, Fedak PW, Verma S (2006) C-reactive protein alters antioxidant defenses and promotes apoptosis in endothelial progenitor cells. Arterioscler Thromb Vasc Biol 26(11):2476–2482
9. Fukazawa R, Ogawa S (2009) Long-term prognosis of patients with Kawasaki disease: at risk for future atherosclerosis? J Nippon Med School 76(3):124–133
10. Furukawa S, Matsubara T, Ichiyama T (2008) Regulation of proinflammatory cytokine cascade in Kawasaki disease. Nippon Rinsho 66(2):258–264
11. He T, Smith LA, Harrington S, Nath KA, Caplice NM, Katusic ZS (2004) Transplantation of circulating endothelial progenitor cells restores endothelial function of denuded rabbit carotid arteries. Stroke 35(10):2378–2384
12. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA et al (2003) Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 348(7):593–600
13. Hui-Yuen JS, Duong TT, Yeung RS (2006) TNF-alpha is necessary for induction of coronary artery inflammation and aneurysm formation in an animal model of Kawasaki disease. J Immunol 176(10):6294–6301
14. Kawasaki T (1967) Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children. Arerugi 16(3):178–222
15. Kawasaki T, Kosaki F, Okawa S, Shigematsu I, Yanagawa H (1974) A new infantile acute febrile mucocutaneous lymph node syndrome (MLNS) prevailing in Japan. Pediatrics 54(3):271–276
16. Lau AC, Duong TT, Ito S, Wilson GJ, Yeung RS (2009) Inhibition of matrix metalloproteinase-9 activity improves coronary outcome in an animal model of Kawasaki disease. Clin Exp Immunol 157(2):300–309
17. Lau AC, Duong TT, Ito S, Yeung RS (2009) Intravenous immunoglobulin and salicylate differentially modulate pathogenic processes leading to vascular damage in a model of Kawasaki disease. Arthritis Rheum 60(7):2131–2141
18. Matsubara T, Furukawa S, Yabuta K (1990) Serum levels of tumor necrosis factor, interleukin 2 receptor, and interferon-gamma in Kawasaki disease involved coronary-artery lesions. Clin Immunol Immunopathol 56(1):29–36
19. Mitani Y, Sawada H, Hayakawa H, Aoki K, Ohashi H, Matsumura M et al (2005) Elevated levels of high-sensitivity C-reactive protein and serum amyloid A late after Kawasaki disease: association between inflammation and late coronary sequelae in Kawasaki disease. Circulation 111(1):38–43
20. Musunuru K, Kral BG, Blumenthal RS, Fuster V, Campbell CY, Gluckman TJ et al (2008) The use of high-sensitivity assays for C-reactive protein in clinical practice. Nat Clin Pract Cardiovasc Med 5(10):621–635
21. Nabata A, Kuroki M, Ueba H, Hashimoto S, Umemoto T, Wada H et al (2008) C-reactive protein induces endothelial cell apoptosis and matrix metalloproteinase-9 production in human mononuclear cells: implications for the destabilization of atherosclerotic plaque. Atherosclerosis 196(1):129–135
22. Niboshi A, Ozawa S, Hamaoka K (2008) Kawasaki disease and atherosclerosis. Nippon Rinsho 66(2):387–392
23. Pinna GS, Kafetzis DA, Tselkas OI, Skevaki CL (2008) Kawasaki disease: an overview. Curr Opin Infect Dis 21(3):263–270
24. Tatara K, Kusakawa S, Itoh K, Kazuma N, Lee K, Hashimoto K et al (1989) Long-term prognosis of Kawasaki disease patients with coronary artery obstruction. Heart Vessels 5(1):47–51
25. Terai M, Shulman ST (1997) Prevalence of coronary artery abnormalities in Kawasaki disease is highly dependent on gamma globulin dose but independent of salicylate dose. J Pediatr 131(6): 888–893
26. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T et al (2002) Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 105(25):3017–3024
27. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M et al (2003) Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res 93(2):e17–e24
28. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A et al (2005) Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 353(10):999–1007
29. Xu MG, Wang JM, Chen L, Wang Y, Yang Z, Tao J (2009) Berberine-induced upregulation of circulating endothelial progenitor cells is related to nitric oxide production in healthy subjects. Cardiology 112(4):279–286
30. Xu MG, Men LN, Zhao CY, Zhao X, Wang YX, Meng XC et al (2010) The number and function of circulating endothelial progenitor cells in patients with Kawasaki disease. Eur J Pediatr 169(3):289–296
31. Zhang Y, Herbert BS, Rajashekhar G, Ingram DA, Yoder MC, Clauss M et al (2009) Premature senescence of highly proliferative endothelial progenitor cells is induced by tumor necrosis factor-alpha by way of the p38 mitogen-activated protein kinase pathway. Faseb J 23(5):1358–1365