Endothelial progenitor cells (CD34$^+$KDR$^+$) and monocytes may provide the development of good coronary collaterals despite the vascular risk factors and extensive atherosclerosis

Endotelyal progenitor hücreler (CD34$^+$KDR$^+$) ve monositler vasküler risk faktörleri ve yaygın ateroskleroza rağmen iyi koroner kollateral gelişimini sağlayabilirler

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ABSTRACT

Objective: Endothelial progenitor cells (EPC) have a regenerative role in the vascular system. In this study, we aimed to evaluate simultaneously the effects of EPC and inflammatory cells on the presence and the extent of coronary artery disease (CAD) and the grade of coronary collateral growth in patients with clinical suspicion of CAD.

Methods: This study has a cross-sectional and observational design. We enrolled 112 eligible patients who underwent coronary angiography consecutively (mean age: 59±9 years). The association of circulating inflammatory cells and EPC (defined by CD34$^+$KDR$^+$ in the lymphocyte and monocyte gate) with the presence, severity and extent of CAD and the degree of collateral growth were investigated. Logistic regression analysis was used to define the predictors of collateral flow.

Results: Of 112 patients 30 had normal coronary arteries (NCA, 27%, 55±9 years) and 82 had CAD (73%, 61±8 years). Among the patients with CAD, the percent degree of luminal stenosis was <50% in 12 patients; 50-90% in 35 patients; and ≥90% in the other 35 patients. Circulating inflammatory cells were higher (leukocytes, 7150±1599 vs 8163±1588mm^{-3}, p=0.001; neutrophils, 4239±1280 vs 4827±1273mm^{-3}, p=0.021; monocytes, 512±111 vs 636±192mm^{-3}, p=0.001) and EPCs were lower (0.27±0.15% vs 0.17±0.14%, p<0.001; 21±15 vs 13±12mm^{-3}, p=0.004) in CAD group than NCA group. When we investigated the collateral growth in patients having ≥90% stenosis in at least one major coronary artery, we found that the patients with good collateral growth had significantly higher EPC (0.22±0.17% vs 0.10±0.05%, p=0.009; 18±15 vs 7±3mm^{-3}, p=0.003) in comparison to patients with poor collateral growth. Presence of EPC was associated with reduced risk for coronary artery disease (OR: 0.934, 95%CI: 0.883-0.998, p=0.018) and was an independent predictor for good collateral growth (OR: 1.295, 95%CI: 1.039-1.615, p=0.022). A sum of CD34$^+$KDR$^-$, CD34$^+$KDR$^+$ and CD34$^-$KDR$^+$ cells (192±98mm^{-3}), and a CD34$^-$KDR$^-$ cell subpopulation within monocyte gate (514±173mm^{-3}) reached to highest counts in good collateral group among all study population.

Conclusion: Endothelial progenitor cells can be mobilized from bone marrow to induce the coronary collateral growth in case of myocardial ischemia even in presence of the vascular risk factors and extensive atherosclerosis. This finding may be supportive to investigate the molecules, which can specifically mobilize EPC without inflammatory cells. *(Anadolu Kardiyol Derg 2011; 11: 290-9)*

Key words: Endothelial progenitor cell, monocyte, collateral development, atherosclerosis, CD34, vascular endothelial growth factor 2, logistic regression analysis

ÖZET

Amaç: Endoteliyal progenitör hücreler (EPH) vasküler sistemde onarıcı bir role sahiptir. Bu çalışmanın amacı kandaki enflamatuvar hücreler ve EPH'lerin, kardiyovasküler risk faktörleri ile birlikte ateroskleroz varlığı ve yaygınlığı ile ilişkisinin araştırılması ve koroner kollateral gelişim üzerine olan etkilerinin incelenmesidir.

Yöntemler: Bu çalışma enine-kesitli ve gözlemsel bir modele sahiptir. Çalışmaya koroner anjiyografisi yapılan ardışık 112 hasta alındı (ortalama yaş: 59±9 yıl). Periferik kanda dolaşan enflamasyon hücreleri ve EPH (lenfositer ve monositer alanda CD34$^+$KDR$^+$ olarak tanımlanan) hücrelerin ateroskleroz varlığı, ciddiyeti, yaygınlığı ve kollateral gelişimi ile olan ilişkileri araştırıldı. Kollateral akım öngördürücülerinin belirlenmesinde lojistik regresyon analizi kullanıldı.

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Bulgular: Yüz on iki hastanın 30'unda normal koroner arterler (NKA, %27, 55±9 yıl), 82'sinde koroner arter hastalığı bulunduğu saptandı (KAH, %73, 61±8 yıl). Koroner arter hastalığı saptanan hastalar arasında 12 hastanın koroner arterinde <%50 darlık, 35 hastada %50-90 arası darlık ve diğer 35 hastada ≥%90 darlık saptandı. Koroner arter hastalığı olan hastaların periferik dolaşımında normal koronerleri olanlara göre daha yüksek inflamatuvar hücre olduğu (Lökosit, 7150±1599'a karşın 8163±1588 mm^{-3}, p=0.001; Nötrofil, 4239±1280'e karşın 4827±1273 mm^{-3}, p=0.021; Monosit, 512±111'e karşın 636±192 mm^{-3}, p=0.001) ve daha düşük EPH'si olduğu (%0.27±0.15'e karşın %0.17±0.14, p<0.001 ve 21±15'e karşın 13±12 mm^{-3}, p=0.004) saptandı. Kollateral gelişim en az bir ana epikardiyal koroner arterinde ≥%90 darlığı olan hastalarda değerlendirildiğinde, iyi kollateral gelişime sahip olan hastalar zayıf kollateral gelişimi olan hastalara göre anlamlı olarak daha yüksek EPH'sine sahipti (%0.10±0.05'e karşın %0.22±0.17, p=0.009; 7±3'e karşın 18±15 mm^{-3}, p=0.003). EPH koroner arter hastalığı riskin azalması ile ilişkili idi (OR: 0.934, %95 GA: 0.883-0.998, p=0.018) ve iyi kollateral gelişim için pozitif bağımsız öngörücüydü (OR: 1.295, %95 GA: 1.039-1.615, p=0.022). Tüm çalışma popülasyonu içerisinde $CD34^+KDR^-$, $CD34^+KDR^+$ ve $CD34^-KDR^+$ hücre toplamı (192±98mm^{-3}) ve monositik alanda bakılan $CD34^-KDR^-$ alt hücre popülasyonu (514±173mm^{-3}) iyi kollateral gelişim grubunda en yüksek değerlerine ulaştı.
Sonuç: Endoteliyal progenitör hücreler miyokardiyal iskemi durumunda koroner kollateral gelişimi uyarmak için vasküler risk faktörleri ve yaygın ateroskleroz varlığında bile kemik iliğinden mobilize edilebilirler. Bu bulgu diğer inflamatuvar hücreleri kemik iliğinden mobilize etmeden spesifik olarak EPH'lerin mobilizasyonunu uyaracak moleküllerin bulunması için bir dayanak olabilir. *(Anadolu Kardiyol Derg 2011; 11: 291-300)*
Anahtar kelimeler: Endotelyal progenitör hücreler, monosit, kollateral gelişim, ateroskleroz, CD34, vasküler endoteliyal büyüme faktörü 2, lojistik regresyon analizi

Introduction

Atherosclerosis is a chronic inflammatory disease, which develops as a process occurring in vessel wall, which begins with response to endothelial injury. Endothelial dysfunction is characterized with dysfunction and loss of monolayer cells covering the inside of the vessels, which is endothelium. Endothelial dysfunction is the first stage in atherosclerosis. The regenerative capacity of endothelium provides protection against atherosclerosis. Failure of the endothelial repair initiates atherosclerotic inflammation and lesion formation, so-called plaque, especially in non-laminar flow stress points in vascular bed (1).

For a long time in vascular system, it is believed that the damaged endothelial cells can only be repaired or replaced by the proliferation and migration of neighboring endothelial cells (2). However, this concept has changed together with determination of endothelial progenitor cells (EPC) having both of stem cell and endothelial cell markers and being able to transform into the endothelial phenotype (3-6).

Coronary angiogenesis and collateral growth are chronic adaptations to myocardial ischemia to restore coronary blood flow and salvage myocardium in the ischemic region. Coronary collateral development has potential protective roles such as limited infract size, less aneurysm formation in the ventricle wall, improved ventricular function, fewer future cardiovascular events and improved survival in patients with occlusive coronary lesions (7, 8).

Endothelial progenitor cells have reparative features in vasculature and are the new aspect of collateral growth. Matsuo et al. (9) investigated whether or not number and function of EPCs were associated with the development of collateral formation in patients with single-vessel coronary artery disease (CAD) of chronic total occlusion and found that EPC-mediated angiogenesis might be associated with coronary collateral formation in humans. Lambiasi et al. (10) suggested that inadequate coronary collateral development is associated with reduced numbers of circulating EPCs in patients with isolated left anterior descending coronary artery disease.

Though few studies have reported the association of EPC with collateral growth, the role of EPC in collateral growth in multivessel CAD with multiple risk factors and their simultaneous association with inflammatory cells, especially monocytes has not been yet established.

In this study, we aimed to evaluate the relation of EPC and inflammatory cells with the presence and extent of CAD and the grade of coronary collateral development in patients with clinical suspicion of CAD.

Methods

This study has a cross-sectional and observational design. One hundred and twelve eligible outpatients who underwent coronary angiography with a suspicion of CAD at the Gazi University Departments of Cardiology between May 2008 and December 2008 were consecutively enrolled in this study. The local Ethics Committee of Gazi University Medical School has approved this study. All the patients gave written informed consent. Clinical characteristics, which consisted of multiple descriptors from each patient's history and physical examination, were collected by physicians from cardiology laboratory for each patient at the time of cardiac catheterization and were stored in the database of coronary angiography laboratory at our institution.

Patients with symptomatic peripheral vascular disease (transient ischemic attack, stroke, intermittent claudication, peripheral revascularization, or amputation), non-ischemic dilated cardiomyopathy, with evidence of ongoing infection or inflammation, recent acute coronary syndrome either with or without ST-segment elevation (at most one month before enrollment), hematological disorders and known malignancy were excluded from the study.

Coronary angiography and collateral vessel development

The extent and the severity of the coronary lesions

Standard selective coronary angiography with at least 4 views of the left coronary system and 2 views of the right coronary artery was performed to all patients using the Judkins technique. Gensini score which considers both the extent and the severity of the lesions at coronary angiography was calculated for each patient (11). This scoring system grades the stenosis in the epicardial coronary arteries (1 for 1-25% stenosis, 2 for 26-50% stenosis, 4 for 51-75% stenosis, 8 for 76-90% stenosis, 16 for 91-99% stenosis, and 32 for total occlusion) and multiplies this number by a constant number determined according to the anatomical position of the lesion.

Determination of coronary collateral development

We investigated the relation of circulating inflammatory cell and EPC with collateral vessel growth in the patients who had ≥ 90% stenosis in at least one major coronary artery. These patient's coronary angiograms were reevaluated for collateral development by two experienced interventional cardiologists who were totally blind to the study. Collateral grading was performed to the vessel with coronary artery stenosis of ≥90% and if the patient had more than one vessel with high-grade stenosis and collateral development; collateral grading had been defined according to vessel that had better collateral. In patients with previous coroner artery bypass grafting (CABG) operation history, if CABG grafts were diseased, it was considered as a lesion in related native vessel.

The grade of coronary collateral development was determined according to the Cohen-Rentrop (12) method: grade 0, no filling of any collateral vessels; grade 1, filling of side branches of the artery to be perfused by collateral vessels without visualization of epicardial segment; grade 2, partial filling of the epicardial artery by collateral vessels; and grade 3, as complete filling of epicardial artery by collateral vessel. Patients with grade 0-1 collateral development were regarded as poor collateral group and patients with grade 2-3 collateral development were regarded as good collateral group.

Identification and quantification of circulating EPC by flow cytometry

Blood samples were drawn by venipuncture before coronary angiography. Fasting venous blood was collected in tubes with EDTA and processed within 2 hours of collection. Phycoerythrin (PE)-labeled anti-CD34 was obtained from Antibodies Direct (AbD) Serotec (Immunoglobulin G1 [IgG1]-PE) (AbD Serotec, Kidlington, UK), allophycocyanin (APC)-labelled anti-kinase domain receptor (KDR) from R&D systems (IgG1-APC) (R&D Systems Europe, Abingdon, UK) and incubation was performed following the manufacturer's instructions. All samples were pretreated with Fc receptor blocking reagent (Sigma, Saint Louis, MO, USA) for 15 minutes at room temperature to prevent non-specific binding of antibodies. For the analysis of the samples, 100 μl of whole blood was incubated with anti-KDR-APC (10μl) and anti-CD34-PE (10μl) for 30 minutes at room temperature. Incubation was followed by erythrocyte lysis (BD FACS Lysing Solution, BD Biosciences, San Jose, CA, USA) and washing in phosphate buffered saline (PBS). Flow cytometry measurement was performed using appropriate fluorescence compensation and setting for lysed whole blood excluding debris and platelets and the number of $CD34^+$ and $CD34^+/KDR^+$ cells were analyzed in the lymphocyte and monocyte gates (mononuclear cells). At least 10.000 events were measured within the myelomonocytic gate. Respective PE- or APC-conjugated isotype control antibodies from the same manufacturers served as controls. Cells were measured using appropriate fluorescence compensation and light scatter gating in a FACSCalibur flow cytometry (Becton Dickinson, USA). Analysis was done using fluorescence-1/fluorescence-2 dot plot quadrant statistics and manual gating (Cell Quest Pro software, Becton Dickinson, BD Biosciences, San Diego, CA, USA) by a blinded approach about patient characteristics. The percentage of positive cells was converted into absolute numbers of cells/mm^{-3} using the white blood cell (WBC) count and the percentages of lymphocytes and monocytes obtained from an automated cell counter (Coulter Gen-S, COULTER Corp, Miami, USA). (Formula 1: Absolute cell count=EPC %totalxWBC/100, Formula 2: Absolute cell count=EPC %gatedx%GatexWBC/10.000).

Routine laboratory measurements

Blood samples were drawn by venipuncture to perform routine blood chemistry after fasting for at least 8 hours before coronary angiography. Fasting blood glucose, serum creatinine, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride levels were recorded. Glucose, creatinine, and lipid profile were determined by standard methods. The Friedewald's formula was used for LDL cholesterol measurement (13). When triglyceride level exceeded 400mg/dl, the direct measurement technique was used for LDL measurement.

Statistical analysis

The SPSS statistical software (SPSS 15.0 for Windows, Inc., Chicago, IL, USA) was used for all statistical calculations. Continuous variables are given as mean±standard deviation; categorical variables ware defined as percentages. Continuous variables were compared by Mann-Whitney U test and the Chi-square test was used for comparison of categorical variables between two groups. Logistic regression analysis was used to determine independent predictors of coronary artery disease and collateral flow.

Age, hypertension, fasting blood glucose, leukocytes and subtypes and EPC were included as independent variables in the logistic regression model to predict the CAD (dependent variable). Patients with good collateral development were excluded from this analysis because of it was an uncorrectable confounding factor for this analysis.

Age, EPC and total coronary occlusion were included as independent variables in the logistic regression model to predict good coronary flow (dependent variable).

All tests of significance were two-tailed. Statistical significance was defined as $p<0.05$.

Results

Endothelial progenitor cells, inflammatory cells and coronary artery disease

The mean age of the patients was 59±9 years. Thirty of 112 consecutive patients had normal coronary artery (NCA, 27%) and 82 had CAD (73%). Among the patients with CAD, the percent degree of luminal stenosis was <50% in 12 patients; 50-90% in 35 patients; and ≥90% in the other 35 patients. Table 1 and 2 show the clinical and biochemical characteristics of the patients.

Circulating inflammatory cells were higher (leukocytes, p=0.001; neutrophils, p=0.021; monocytes, p=0.001) (Table 1), and $CD34^+$cells (cell percent, p<0.001; cell count, p=0.005) and $CD34^+KDR^+$ cells (cell percent, p<0.001; cell count, p=0.004) (Table 1, 3; Fig. 1) were lower in CAD group than NCA group.

Age (OR=1.107, 95%CI (1.014-1.209), p=0.024), leukocytes, especially neutrophils (OR=1.001, 95%CI 1.000-1.001, p=0.009) were positive independent predictors and EPC (OR=0.934, 95%CI 0.883-0.998, p=0.018) was negative independent predictor for CAD (Table 4).

Endothelial progenitor cells, monocytes and collateral development

We also found that patients with good collateral growth had significantly higher EPC (cell percent, p=0.009; cell count, p=0.003) (Table 2) in comparison to patients with poor collateral growth. A sum of $CD34^+KDR^-$, $CD34^+KDR^+$ and $CD34^-KDR^+$cells (192±98 mm^{-3}) (Fig. 2) and a $CD34^-KDR^-$ cell subpopulation within monocyte gate (514±173 mm^{-3}) reached to highest counts in good collateral group in our study (Fig. 3).

In logistic regression analysis among all independent variables like age, EPC and total coronary occlusion, only EPC was an independent predictor for good collateral growth (OR=1.295, 95%CI 1.039-1.615, p=0.02) (Table 5).

Discussion

In this study, we aimed to evaluate the effects of progenitor and inflammatory cells on CAD and coronary collateral growth simultaneously. Our findings suggested that EPC is an independent predictor for coronary collateral formation despite of extensive atherosclerosis and cardiovascular risk factors. In additionally, a specific subpopulation of monocytes, which were not included the progenitors was related to good collaterals. On the other hand, EPCs reduced risk for CAD while inflammatory cells, especially neutrophils had incremental effect on CAD.

During the last half century, great advances in the treatment of acute and chronic forms of atherosclerosis have been achieved. These advances were provided by controlling of the offended risk factors for atherosclerosis and, by using evidence based drugs and devices for its clinical manifestations. Together with these advances, today additional improvement cannot be provided on reached event reduction rates for treatment of stable CAD. This treatment resistance can be broken by discovery of new cells, cytokines, receptors and regulators in vascular system.

The role of endothelium is beyond to be only a cell monolayer inside vessels. It has been understood with recognition of the novel parameters that represent endothelium health status and independently predict the all-vascular events. However, like many mature cell lines, endothelial cells have limited reparative ability, especially in pathological microenvironments produced by vascular risk factors.

Recently, it was proved that endothelium is not alone in compensation for the damaging effect of cardiac risk factors in vasculature. In this reparative process, a more important role belongs to EPC in circulation. After first time defined by Asaraha et al. (14), we have more knowledge about their source, roles, levels and functionality. Today, the treatment potential of these cells for atherosclerosis is an important research area. Different cell types and application routes are under active search for cardiac regeneration (15).

Coronary angiogenesis and collateral growth are chronic adaptations to myocardial ischemia to restore coronary blood flow and salvage myocardium in the ischemic region. Several contributing factors have been reported in relation to collateral development. The severity of coronary artery stenosis and the duration of myocardial ischemic symptoms have been found in association with good collateral formation (16, 17). Patients with diabetes, hypercholesterolemia, and hypertension have less ability to create collateral vessels (18-20). Myocardial infarction and revascularization procedures may cause to decrease visible collaterals.

In a recent study related to collateral development, various cytokines were studied but insufficient results were obtained (21). Significant relationship has been found only between monocyte functions and monocyte transcription profiling with good collateral development (22, 23). Heterogeneity in collateral formation despite similar degrees of coronary obstruction may be related to several factors such as different effects of inflammatory cells, the capability of cell homing factors in the ischemic tissue and levels of both cytokines and chemokines related with ischemic tissue. The quantity and quality of functional cells may be critical in the development of collaterals. Besides, these stages may be operative by undefined mechanisms such as other cells, cytokines and receptors that contribute to inflammation process.

Previous experimental animal studies also demonstrated that monocytes could be important elements for development of collateral vessels (24-26). In 1976, Schaper et al. (25) demonstrated the histological evidence for monocyte adhesion and migration to the endothelium of newly developing collateral arteries in dog hearts. More recently, in functional studies, which were done in animals, arteriogenesis has been shown to correlate directly with the concentration of circulating monocytes and the amount of accumulating monocytes/macrophages in the perivascular tissue (26).

Table 1. Baseline demographic, biochemical and hematological parameters in patients with normal coronary artery and coronary artery disease

Variables	NCA (n=30)		CAD (n=82)		p*
	Mean±SD	Median (min-max)	Mean±SD	Median (min-max)	
Age, years	55±9	54 (30-70)	61±8	61 (42-79)	0.010
Gender, male, %	60		77		0.078
Systolic BP, mmHg	126±17	125 (95-165)	131±21	130 (90-180)	0.331
Diastolic BP, mmHg	80±10	80 (60-100)	79±10	80 (60-100)	0.531
Hypertension, %	35		61		0.030
Diabetes mellitus, %	17		39		0.055
Family history of CAD, %	26		32		0.622
Smoking, current, %	13		20		0.674
EF, %	65±5	66 (46-72)	56±13	61 (22-72)	<0.001
The distribution of diseased coronary vessels, n(%)					
Coronary luminal narrowing <50%	12 (15)				
50-90%	35 (43)				
≥90%	35 (43)				
100%	22 (27)				
Biochemistry					
Fasting blood glucose, mg/dl	98±13	99 (75-138)	120±45	103 (51-271)	0.043
Creatinine, mg/dl	0.9±0.2	0.9 (0.6-1.3)	1.2±1.1	1 (0.6-7.1)	0.373
Total cholesterol, mg/dl	186±34	187 (130-272)	187±50	183 (21-321)	0.966
LDL, mg/dl	118±31	117 (55-194)	116±40	108 (58-246)	0.482
HDL, mg/dl	43±9	42 (23-58)	42±11	40 (25-89)	0.245
Triglyceride, mg/dl	142±74	121 (49-412)	157±79	147 (2-461)	0.303
Complete blood count (CBC)					
Hemoglobin, mg/dl	14.6±1.2	14.7 (12.5-17)	14.2±1.3	14 (9.2-16.8)	0.136
Platelets, 10^3/mm^{-3}	231±49	228 (157-355)	230±78	223 (114-695)	0.466
Leukocytes, mm^{-3}	7150±1599	7040 (4730-11600)	8163±1588	8435 (4510-10900)	0.001
Neutrophils, mm^{-3}	4239±1280	3970 (2660-8560)	4827±1273	4820 (2610-7890)	0.021
Lymphocytes, mm^{-3}	2170±683	2190 (1350-3870)	2178±795	2340 (736-4370)	0.392
Monocytes, mm^{-3}	512±111	504 (343-997)	636±192	623 (226-1300)	0.001
Eosinophils, mm^{-3}	145±73	129 (25-399)	230±242	170 (11-1860)	0.118
Flow cytometry (FACS)					
$CD34^+$ cell, %	0.72±0.34	0.7 (0.2-1.4)	0.48±0.30	0.4 (0.1-1.8)	<0.001
$CD34^+$ cell, mm^{-3}	52±26	47 (9-112)	39±27	33 (8-187)	0.005
$CD34^+KDR^+$ cell, %	0.27±0.15	0.2 (0.07-0.63)	0.17±0.14	0.1 (0-0.7)	<0.001
$CD34^+KDR^+$ cell, mm^{-3}	21±15	17 (3.3-49)	13±12	11 (0-52)	0.004
KDR^+ cell, %	1.6±1.0	1.5 (0.3-4.2)	1.5±1.0	1.5 (0.1-4.2)	0.574
KDR^+ cell, mm^{-3}	117±73	113 (18-312)	124±94	104 (9-459)	0.989
Medications					
ASA, %	33		79		0.016
Beta blockers, %	44		70		0.123
ACEi/ARB, %	22		56		0.061
Statin, %	22		54		0.079
Oral anti-diabetic, %	11		20		0.513

Data are presented as mean±standard deviation, median (min-max) and percentages

*Mann-Whitney U and Chi-square tests

ACEI- angiotensin converting enzyme inhibitor, ARB- angiotensin II receptor blocker, ASA- acetyl salicylic acid, BP- blood pressure, CAD- coronary artery disease, CD34- cluster domain 34, EF- ejection fraction, FACS- fluorescence-activated cell sorting, HDL- high-density lipoprotein, KDR- kinase insert domain receptor, LDL- low-density lipoprotein, NCA- normal coronary artery

Table 2. Baseline demographic, biochemichal and hematological parameters in collateral growth Rentrop groups

Variables**	Poor collateral growth, Rentrop 0.1 (n=12)		Good collateral growth, Rentrop 2.3 (n=23)		p*
	Mean±SD	Median (min-max)	Mean±SD	Median (min-max)	
Age, years	61±10	59 (50-79)	60±8	61 (42-74)	0.931
Gender, male, %	75		87		0.373
Systolic BP, mmHg	123±18	120 (100-160)	138±22	135 (110-180)	0.078
Diastolic BP, mmHg	74±10	75 (60-90)	82±11	80 (70-100)	0.127
Hypertension, %	42		74		0.075
Diabetes mellitus, %	42		55		0.465
Family history of CAD, %	50		28		0.216
Smoking, current, %	25		39		0.125
MI history, %	40		40		1.000
The time of first diagnosis of CAD, years	5±3		5±4		0.996
CABG, %	25		27		0.886
The time of previous CABG, years	3±5		4±3		0.250
Gensini score	43±33	34 (12-106)	59±29	59 (18-110)	0.094
EF, %	59±11	65 (35-70)	53±12	56 (30-67)	0.060
Total coronary occlusion, n	5		17		0.020
Biochemistry					
Fasting blood glucose, mg/dl	135±57	102 (79-271)	131±44	121 (84-237)	0.931
Creatinine, mg/dl	1.0±0.2	1 (0.8-1.3)	1.4±1.5	1 (0.6-7)	0.862
Total cholesterol, mg/dl	180±41	184 (123-250)	186±47	176 (122-311)	0.971
LDL, mg/dl	106±34	109 (61-178)	116±43	107 (59-230)	0.885
HDL, mg/dl	46±16	42 (33-89)	40±5	39 (28-49)	0.481
Triglyceride, mg/dl	141±70	114 (53-253)	165±80	150 (52-461)	0.397
Complete blood count					
Hemoglobin, mg/dl	14±1	14 (12-15)	14±2	14 (9-16)	0.957
Platelets, 10^3/mm^{-3}	211±29	217 (158-249)	240±64	228 (114-380)	0.230
Leukocytes, mm^{-3}	7805±1914	8435 (4510-10400)	8315±1473	8030 (6050-10600)	0.664
Neutrophils, mm^{-3}	4560±1563	4690 (2610-7890)	4949±1178	4750 (3340-7160)	0.305
Lymphocytes, mm^{-3}	2398±1003	2290 (736-3980)	2409±655	2450 (1500-4370)	0.754
Monocytes, mm^{-3}	548±159	620 (271-796)	664±219	674 (226-1300)	0.164
Eosinophils, mm^{-3}	233±195	138 (17-532)	223±136	196 (11-490)	0.885
Flow cytometry (FACS)					
CD34$^+$ cell, %	0.35±0.13	0.3 (0.2-0.7)	0.61±0.44	0.5 (0.1-1.8)	0.014
CD34$^+$ cell, mm^{-3}	26±8	25 (16-42)	51±42	37 (12-187)	0.012
CD34$^+$KDR$^+$ cell, %	0.10±0.05	0.1 (0.04-0.2)	0.22±0.17	0.2 (0.03-0.7)	0.009
CD34$^+$KDR$^+$ cell, mm^{-3}	7±3	6.8 (3-16)	18±15	13 (2-52)	0.003
KDR$^+$ cell, %	1.33±1.0	1.1 (0.1-2.9)	1.89±1.0	1.9 (0.3-4)	0.126
KDR$^+$ cell, mm^{-3}	99±84	75 (11-241)	159±93	162 (17-312)	0.071
Medications					
ASA, %	78		77		0.962
Beta blockers, %	56		62		0.779
ACEi/ARB, %	44		39		0.779
Statin, %	44		69		0.245
Oral anti-diabetic, %	22		31		0.658

Data are presented as mean±standard deviation, median (min-max) and percentages

*Mann-Whitney U and Chi-square tests

**The relation of circulating inflammatory cell and EPC with collateral vessel growth was searched in the patients who had ≥ 90% stenosis in at least one major coronary artery

ACEI- angiotensin converting enzyme inhibitor, ARB- angiotensin II receptor blocker, ASA- acetyl salicylic acid, BP- blood pressure, CABG- coronary bypass surgery, CAD- coronary artery disease, CD34- cluster domain 34, EF- ejection fraction, FACS- fluorescence-activated cell sorting, HDL- high-density lipoprotein, KDR- kinase insert domain receptor, LDL- low-density lipoprotein, MI- myocardial infarction, NCA- normal coronary artery

Table 3. Comparison of leukocyte and subtypes with CD34+, CD34+KDR+, KDR+ cells in study subgroups

Variables	Study subgroups						
	NCA	CAD			Rentrop		p^R
		<50%	50-90%	≥90%	0/1 (Poor)	2/3 (Good)	
	(n=30)	(n=12)	(n=35)	(n=35)	(n=12)	(n=23)	
Leukocytes, mm^{-3}	7121±1617	8276±1702*	8142±1556**	8144±1623**	7805±1914	8315±1473**	0.664
Neutrophils, mm^{-3}	4214±1348	5039±1479	4754±1189	4824±1305*	4560±1563	4949±1178*	0.305
Lymphocytes, mm^{-3}	2191±512	2294±802	2385±673	2403±778	2398±1003	2409±655	0.754
Monocytes, mm^{-3}	512±111	659±164**	646±184**	618±210**	548±159	664±219**	0.164
Eosinophils, mm^{-3}	145±73	229±153	230±330	230±154	233±195	223±136	0.885
$CD34^+$ cell, %	0.72±0.34	0.42±0.19**	0.44±0.23**	0.54±0.38**	0.35±0.13**	0.61±0.44	0.014
$CD34^+$ cell, mm^{-3}	55±36	33±17*	38±22*	44±36*	26±8**	51±42	0.012
$CD34^+KDR^+$ cell, %	0.27±0.15	0.15±0.08*	0.16±0.15**	0.19±0.16**	0.10±0.05***	0.22±0.17	0.009
$CD34^+KDR^+$, mm^{-3}	21±15	11±7	13±11*	15±14*	7±3***	18±15	0.003
KDR^+ cell, %	1.6±1.0	1.5±1.3	1.4±0.9	1.7±1.1	1.33±1.0	1.89±1.0	0.126
KDR^+ cell, mm^{-3}	116±73	121±126	113±84	138±93	101±83	159±93	0.071

Data are presented as mean±standard deviation
*Mann-Whitney U test
When compared with NCA- *p<0.05, **p<0.01, ***p<0.001
R- p value for difference between Rentrop groups
CAD - coronary artery disease, CD34 - cluster domain 34, KDR - kinase insert domain receptor, NCA - normal coronary artery

Table 4. Logistic regression analysis of predictors for coronary artery disease

Independent variables**	Wald	OR	95% Confidence Interval	p*
Age, years	5.1	1.107	(1.014-1.209)	0.024
Hypertension, %	0.133	1.282	(0.337-4.875)	0.716
Fasting blood glucose, mg/dl	0.393	1.007	(0.985-1.029)	0.531
Leukocytes, mm^{-3}	6.5	1.001	(1.000-1.001)	0.011
Neutrophils, mm^{-3}	6.8	1.001	(1.000-1.001)	0.009
Lymphocytes, mm^{-3}	0.1	1.000	(0.999-1.001)	0.803
Monocytes, mm^{-3}	2.7	1.003	(0.999-1.007)	0.103
Eosinophils, mm^{-3}	1.4	1.003	(0.998-1.009)	0.242
EPC, $CD34^+KDR^+$cell, mm^{-3}	5.6	0.934	(0.883-0.998)	0.018
Constant	7.3	0.000	0.000	0.007

*Logistic regression analysis with enter method **Patients with good collateral development were excluded from the analysis because of it is an uncorrectable confounding factor for this analysis
CD34 - cluster domain 34, EPC - endothelial progenitor cell, KDR - kinase insert domain receptor

In previous studies, we showed a positive significant correlation between the monocyte count and collateral development in diabetic (643±184 vs 479±143 mm^{-3}, p<0.001) and non-diabetics (671±218 vs 522±195 mm^{-3}, p<0.001) (27, 28). These findings have suggested that the monocytes may have a key role in the integrity of arteriogenesis even in the clinical setting as well as in experimental studies. If monocytes have reparative functions in atherosclerosis, at least a subpopulation, the insufficiency of monocyte quality and quantity in patients with CAD may be important in all vascular reparative mechanisms.

Table 5. Logistic regression analysis of predictors for good coronary collateral development

Independent variables**	Wald	OR	95% Confidence Interval	p*
EPC, $CD34^+KDR^+$cell, mm^{-3}	5.3	1.295	1.039-1.615	0.022
Total coronary occlusion, n	3.0	4.889	0.811-29.4	0.083
Constant	3.4	0.136	0.136	0.065

*Logistic regression analysis with enter method
CD34 - cluster domain 34, EPC - endothelial progenitor cell, KDR - kinase insert domain receptor

Endothelial progenitor cell is the new aspect of collateral growth. These mononuclear cells derived from bone marrow, have been implicated in the production of new blood vessel development (9, 10). The cells have reparative features in vasculature. Cardiovascular risk factors both attenuate the function and the amount of these cells (29). Vascular progenitor cells are presumably counted within the monocyte population detected by Coulter analysis, and they may contribute to collateral vessel development. Therefore in the current study, we excluded all $CD34^+$ and KDR^+ cell types from monocyte gate, and then when we looked redundant cells, which represented the isolated

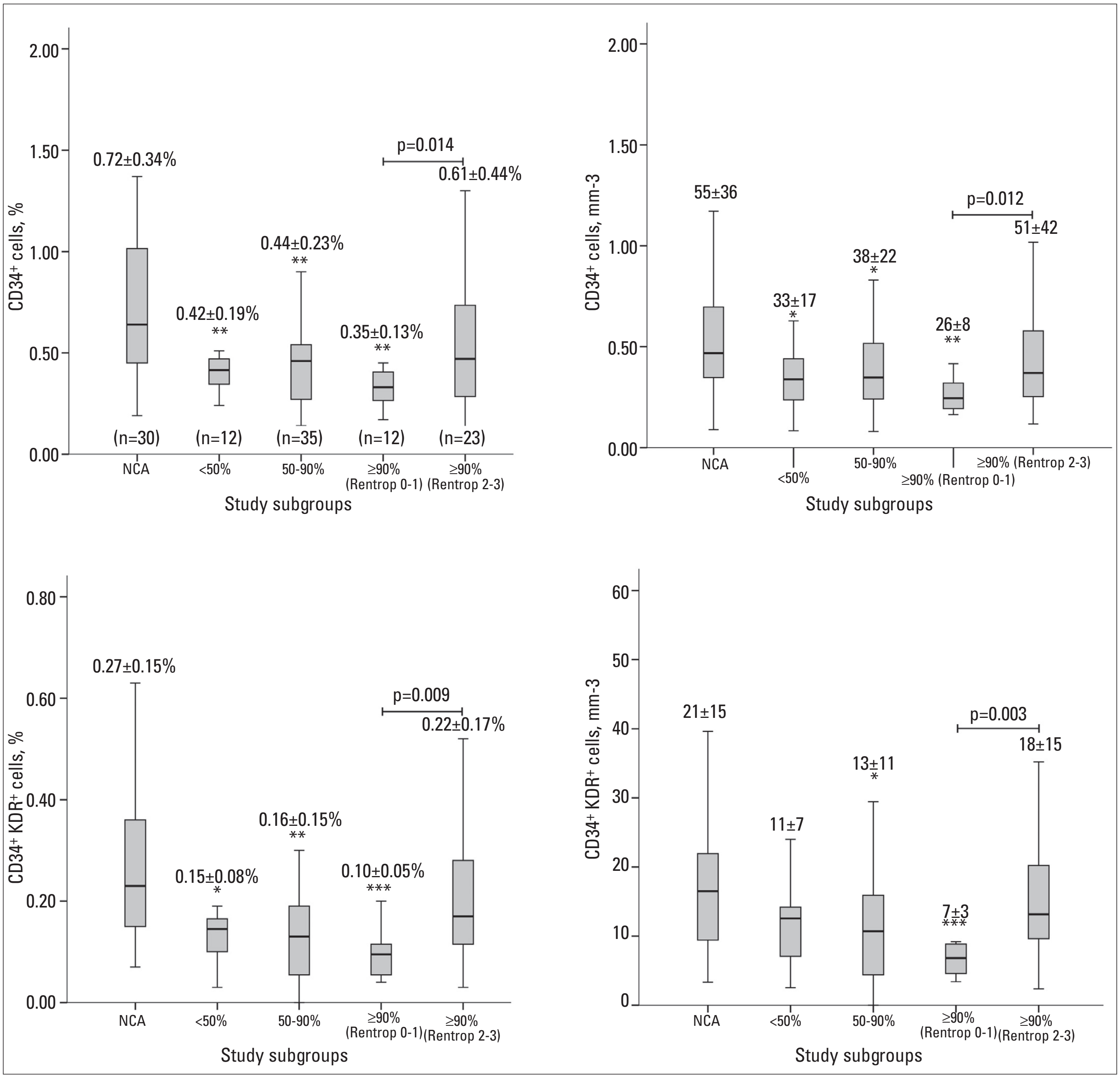

Figure 1. Circulating progenitor cells counts ($CD34^+$ and $CD34^+KDR^+$) in the study subgroups according to severity of CAD
When compared with NCA group: *p<0.05, **p<0.01, ***p<0.001

CAD - coronary artery disease, CD34 - cluster domain 34, KDR - kinase insert domain receptor, NCA - normal coronary artery

$CD34^-KDR^-$ monocytes, we found that patients with good collateral had still highest monocyte count (before exclusion, 664±219 vs 548±159 mm^{-3} ; after exclusion of CD34 cells, 628±210 vs 537±183 mm^{-3}; after exclusion of both CD34 and KDR cells, 514±173 vs 469±162 mm^{-3}). This findings support that there is a specific monocyte subpopulation other than progenitors, which has an independent function in collateral growth. Monocytes and EPCs may have common and/or different roles in the integrity of arteriogenesis.

In our study, we selected patients who underwent angiography consequently and in that way produced a spectrum for CAD. This study design provided opportunity to investigate and to compare the development of CAD and collateral growth at same population. EPC has also been evaluated together with inflammatory cells. While inflammatory cells increase with presence, severity and extent of CAD, progenitor cells decreased. Lowest value of EPC was found in poor collateral subgroup. This finding is of interest because poor and good collateral growth groups

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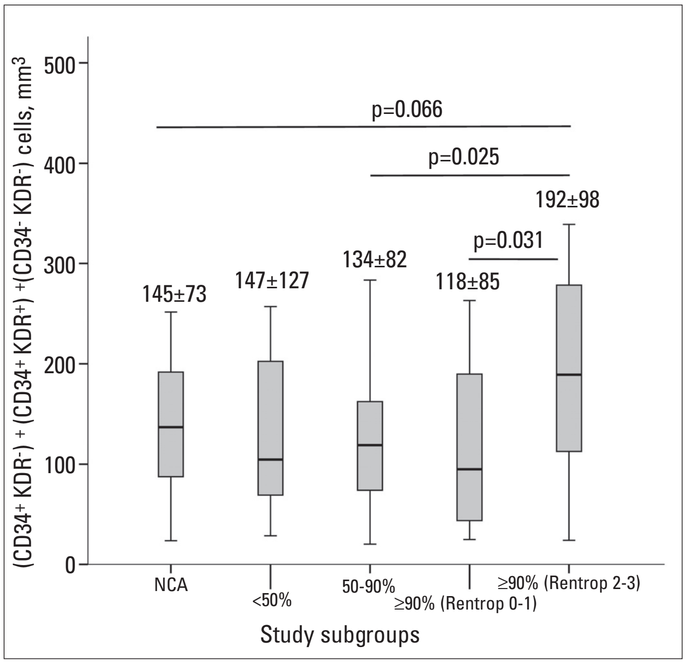

Figure 2. Sum of CD34+KDR-, CD34+KDR+ and CD34-KDR+cells count in study subgroups according to severity of CAD

CAD - coronary artery disease , CD34 - cluster domain 34, KDR - kinase insert domain receptor

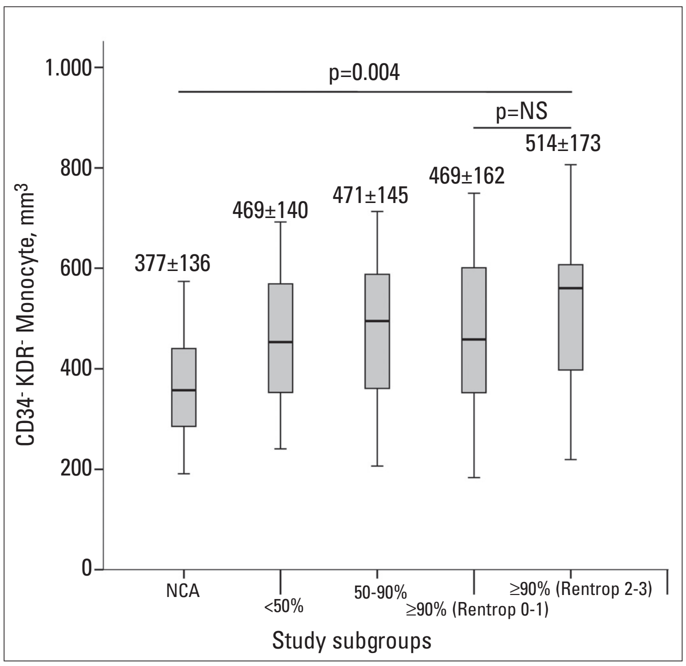

Figure 3. Alteration of CD34-KDR- monocyte subpopulation[a] in study subgroups according to severity of CAD [a] excluded all CD34+ and KDR+ cell types in monocyte gate

CAD - coronary artery disease , CD34 - cluster domain 34, KDR - kinase insert domain receptor

had similar atherosclerotic coronary burden determined by Gensini score and in multivariate analyses, EPC was related with good collaterals independent of chronic total coronary occlusion. Another important finding was that good collateral group has reached similar to the NCA group EPC count (21±15 vs 18±15 mm^{-3}, p=NS). In atherosclerotic process, EPC count and function were found to depress with vascular risk factors, but in current study population, progenitor cells again increased to nearly normal levels in good collateral group by severe coronary ischemia, despite of the severe coronary atherosclerotic burden. This increase in progenitors did not include only EPC, but also other CD34 progenitor and KDR cells too. Moreover, a sum of $CD34^+KDR^-$, $CD34^+KDR^+$ and $CD34^-KDR^+$cells (192±98mm^{-3}) in good collateral group has reached to higher counts than the counts in NCA group (Fig. 2). This finding shows that the response mechanisms to ischemia related with progenitors was still intact in patients with good collateral.

Our findings suggest that the peripheral effect of cardiovascular risk factor on progenitors is not entirely valid for bone marrow, because if this were the case, the decreased progenitor cells would not be able reach to normal levels in good collateral group within patient spectrum of CAD.

Patients with poor collateral had lowest EPC count despite of the presence of significant ischemia. The possible causes of this insufficiency in the response must be explained. In a previous study, we found that collateral growth had inverse relation with asymmetric dimethylarginine (ADMA), which is a biological synthesis blocker of nitric oxide (NO)(30). While this association suggests a critical role for NO on collateral development, it also supports the integral regulator function of endothelium in this process. In our opinion, there is probably a defect in ischemia-induced cytokine generation of endothelium, which affects specifically the bone marrow to mobilize progenitors.

Study limitations

Our study had some limitations. First of all, study population was relatively small. Larger study population would provide higher statistical power. The other one, in vitro cell functions, the cytokines, which are functional for collateral growth in physiologic circumstances, were not studied in this study. This kind of analysis would probably provide additional information on collateral growth and atherosclerosis. Lastly, in our study, control group included the patients who are not completely normal, because although they have angiographically normal coronary arteries they still have cardiac risk factors or may have cardiac syndrome-X. Therefore, the statistical differences would be difficult to determine between normal and pathologic group. Otherwise, our study population proved many significant relations among study groups.

It is known that collateral growth and CAD are long-lasting process and disease. Therefore, it may be thought that one-time measurement cannot represent all courses. Especially this may be true for collateral growth because of it is responsive to coronary ischemia. However, good collateral growth may not totally relieve the coronary ischemia and secondly some patients may individually have higher basal values for progenitor cells which can determine tendency for the atherosclerosis and capability for collateral development.

Conclusion

The stimulation of collateral growth in a safe manner would have an important role in patients with no treatment option. Endothelial progenitor cells can be mobilized from bone marrow to induce the coronary collateral growth in case of myocardial ischemia even in presence of the vascular risk factors and extensive atherosclerosis. This finding may be supportive to investigate the molecules, which can specifically mobilize EPC without inflammatory cells and would be the drug of chose for regenerative medicine in vascular system.

Conflict of interest: None declared.

References

1. Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, Garcia-Cardeña G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. Ann N Y Acad Sci 2000; 902: 230-9.
2. Ross R, Glomset J, Harker L. Response to injury and atherogenesis. Am J Pathol 1977; 86: 675-84.
3. Gulati R, Jevremovic D, Peterson TE, Witt TA, Kleppe LS, Mueske CS, et al. Autologous culture-modified mononuclear cells confer vascular protection after arterial injury. Circulation 2003; 108: 1520-6.
4. He T, Smith LA, Harrington S, Nath KA, Caplice NM, Katusic ZS. Transplantation of circulating endothelial progenitor cells restores endothelial function of denuded rabbit carotid arteries. Stroke 2004; 35: 2378-84.
5. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Wang T, Gregg D, Ramaswami P, et al. Aging, progenitor cell exhaustion, and atherosclerosis. Circulation 2003; 108: 457-63.
6. Wassmann S, Werner N, Czech T, Nickenig G. Improvement of endothelial function by systemic transfusion of vascular progenitor cells. Circ Res 2006; 99: e74-83.
7. Habib GB, Heibig J, Forman SA, Brown BG, Roberts R, Terrin ML, et al. Influence of coronary collateral vessels on myocardial infarct size in humans. Results of phase I thrombolysis in myocardial infarction (TIMI) trial. The TIMI investigators. Circulation 1991; 83: 739-46.
8. Hansen JF. Coronary collateral circulation: clinical significance and influence on survival in patients with coronary artery occlusion. Am Heart J 1989; 117: 290-5.
9. Matsuo Y, Imanishi T, Hayashi Y, Tomobuchi Y, Kubo T, Hano T, et al. The effect of endothelial progenitor cells on the development of collateral formation in patients with coronary artery disease. Intern Med 2008; 47: 127-34.
10. Lambiase PD, Edwards RJ, Anthopoulos P, Rahman S, Meng YG, Bucknall CA, et al. Circulating humoral factors and endothelial progenitor cells in patients with differing coronary collateral support. Circulation 2004; 109: 2986-92.
11. Gensini GG. A more meaningful scoring system for determining the severity of coronary heart disease. Am J Cardiol 1983; 51: 606.
12. Rentrop KP, Cohen M, Blanke H, Phillips RA. Changes in collateral channel filling immediately after controlled coronary artery occlusion by an angioplasty balloon in human subjects. J Am Coll Cardiol 1985; 5: 587-92.
13. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoproteins cholesterol in plasma without use of the ultracentrifuge. Clin Chem 1972; 18: 499-502.
14. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-7.
15. Kaminski A, Steinhoff G. Current status of intramyocardial bone marrow stem cell transplantation. Semin Thorac Cardiovasc Surg 2008; 20:119-25.
16. Kilian JG, Keech A, Adams MR, Celermajer DS. Coronary collateralization: determinants of adequate distal vessel filling after arterial occlusion. Coron Artery Dis 2002; 13: 155-9.
17. Pohl T, Seiler C, Billinger M, Herren E, Wustmann K, Mehta H, et al. Frequency distribution of collateral flow and factors influencing collateral channel development. Functional collateral channel measurement in 450 patients with coronary artery disease. J Am Coll Cardiol 2001; 38: 1872-8.
18. Kornowski R. Collateral formation and clinical variables in obstructive coronary artery disease: the influence of hypercholesterolemia and diabetes mellitus. Coron Artery Dis 2003; 14: 61-4.
19. Kilian JG, Keech A, Adams MR, Celermajer DS. Coronary collateralization: determinants of adequate distal vessel filling after arterial occlusion. Coron Artery Dis 2002; 13: 155-9.
20. Abacı A, Oğuzhan A, Kahraman S, Eryol NK, Ünal S, Arınç H, et al. Effect of diabetes mellitus on formation of coronary collateral vessels. Circulation 1999; 99: 2239-42.
21. Sherman JA, Hall A, Malenka DJ, De Muinck ED, Simons M. Humoral and cellular factors responsible for coronary collateral formation. Am J Cardiol 2006; 98: 1194-7.
22. Chittenden TW, Sherman JA, Xiong F, Hall AE, Lanahan AA, Taylor JM, et al. Transcriptional profiling in coronary artery disease: indications for novel markers of coronary collateralization. Circulation 2006; 114:1811-20.
23. Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W. Monocyte activation in angiogenesis and collateral growth in the rabbit hind limb. J Clin Invest 1998; 101: 40-50.
24. Herold J, Pipp F, Fernandez B, Xing Z, Heil M, Tillmanns H, et al. Transplantation of monocytes: a novel strategy for in vivo augmentation of collateral vessel growth. Hum Gene Ther 2004; 15: 1-12.
25. Schaper J, Konig R, Franz D, Schaper W. The endothelial surface of growing coronary collateral arteries. Intimal margination and diapedesis of monocytes. A combined SEM and TEM study. Virchows Arch A Pathol Anat Histol 1976; 370: 193-205.
26. Heil M, Ziegelhoeffer T, Pipp F, Kostin S, Martin S, Clauss M, et al. Blood monocyte concentration is critical for the enhancement of collateral artery growth. Am J Physiol Heart Circ Physiol 2002; 283:H2411-9.
27. Kocaman SA, Arslan U, Tavıl Y, Okuyan H, Abacı A, Çengel A. Increased circulating monocyte count is related to good collateral development in coronary artery disease. Atherosclerosis 2008; 197: 753-6.
28. Kocaman SA, Şahinarslan A, Akyel A, Timurkaynak T, Boyacı B, Çengel A. The association of circulating monocyte count with coronary collateral growth in patients with diabetes mellitus. Acta Diabetol 2010; 47: 49-54.
29. Shantsila E, Watson T, Lip GY. Endothelial progenitor cells in cardiovascular disorders. J Am Coll Cardiol 2007; 49: 741-52.
30. Kocaman SA, Şahinarslan A, Biberoğlu G, Hasanoğlu A, Akyel A, Timurkaynak T, et al. Asymmetric dimethylarginine and coronary collateral vessel development. Coron Artery Dis 2008; 19: 469-74.