ORIGINAL ARTICLE

Circulating Endothelial Progenitor Cells, Vascular Function, and Cardiovascular Risk

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ABSTRACT

BACKGROUND

Cardiovascular risk factors contribute to atherogenesis by inducing endothelial-cell injury and dysfunction. We hypothesized that endothelial progenitor cells derived from bone marrow have a role in ongoing endothelial repair and that impaired mobilization or depletion of these cells contributes to endothelial dysfunction and cardiovascular disease progression.

METHODS

We measured the number of colony-forming units of endothelial progenitor cells in peripheral-blood samples from 45 men (mean [±SE] age, 50±2 years). The subjects had various degrees of cardiovascular risk but no history of cardiovascular disease. Endothelium-dependent and endothelium-independent function was assessed by high-resolution ultrasonography of the brachial artery.

RESULTS

We observed a strong correlation between the number of circulating endothelial progenitor cells and the subjects' combined Framingham risk factor score (r=−0.47, P=0.001). Measurement of flow-mediated brachial-artery reactivity also revealed a significant relation between endothelial function and the number of progenitor cells (r=0.59, P<0.001). Indeed, the levels of circulating endothelial progenitor cells were a better predictor of vascular reactivity than was the presence or absence of conventional risk factors. In addition, endothelial progenitor cells from subjects at high risk for cardiovascular events had higher rates of in vitro senescence than cells from subjects at low risk.

CONCLUSIONS

In healthy men, levels of endothelial progenitor cells may be a surrogate biologic marker for vascular function and cumulative cardiovascular risk. These findings suggest that endothelial injury in the absence of sufficient circulating progenitor cells may affect the progression of cardiovascular disease.

STUDIES HAVE IDENTIFIED A CELL POPU-lation termed endothelial progenitor cells that can be isolated from circulating mono-nuclear cells,[1-3] bone marrow,[4] and cord blood.[5] Laboratory evidence suggests that these cells express a number of endothelial-specific cell-surface markers and exhibit numerous endothelial properties.[1,3] In addition, when these cells are injected into animal models with ischemia, they are rapidly incorporated into sites of neovascularization.[1,5-11]

Ross's classic paradigm states that endotheli-al-cell injury is the stimulus for the development of atherosclerotic plaque.[12] This model argues that seemingly disparate risk factors act on a final common pathway that culminates in endothelial-cell injury. This paradigm has been modified to include both direct endothelial damage and endothelial dysfunction. Thus, indicators of cumulative risk, such as the Framingham score, or function, such as bra-chial reactivity, represent useful composite measures of overall vascular status. The results of several recent studies have supported this concept by demonstrating that endothelial function is a predictor of the risk of cardiovascular events.[13-17]

We hypothesized that circulating endothelial progenitor cells might contribute to ongoing endothelial repair. In particular, endothelial progenitor cells may provide a circulating pool of cells that could form a cellular patch at the site of denuding injury or serve as a cellular reservoir to replace dysfunctional endothelium. Although earlier studies suggested that, at least in the case of denuding injury, extension of neighboring mature endothelial cells was responsible for repair, there is a growing understanding that endothelial progenitor cells also contribute to this process.[18-20]

To test this hypothesis, we measured the activity of endothelial progenitor cells in relation to cardiovascular risk factors and endothelial function in a group of healthy volunteers. These subjects had no symptoms associated with atherosclerosis or active ischemia.

METHODS

STUDY SUBJECTS

We studied 45 men who were older than 21 years of age (mean [±SE] age, 50±2), some of whom had conventional cardiovascular risk factors and some of whom did not. Subjects were solicited through the Patient Recruitment and Public Liaison Office of the National Institutes of Health. The total bur-den of risk factors was calculated with use of the Framingham risk factor score, which has previously been used to predict the risk of coronary artery disease in persons free of clinical disease.[21] Scores can range from −6 to 19, with higher scores indicating greater cardiovascular risk.

Subjects were excluded from this study if they had known or symptomatic cardiovascular disease or had any condition, such as cancer or retinopathy, in which neovascularization might be present. Similarly, women were excluded from this study because of the potential confounding effects of the limited angiogenesis that occurs during the menstrual cycle.[22,23] All enrolled subjects underwent a detailed assessment of cardiovascular risk after signing an informed consent document approved by the institutional review board of the National Heart, Lung, and Blood Institute.

No medications, including vitamins, were taken for at least one week before the study. Statins and angiotensin-converting–enzyme (ACE) inhibitors were discontinued two months before the study began, after appropriate tapering of the dose, and other antihypertensive medications were discontinued at least two weeks before the study with appropriate blood-pressure monitoring. Subjects with diabetes continued their regular glucose-control medications.

ISOLATION OF ENDOTHELIAL PROGENITOR CELLS AND COLONY-FORMING ASSAY

A 20-ml sample of venous blood was used for the isolation of endothelial progenitor cells. Samples were processed within four hours after collection, and peripheral-blood mononuclear cells were isolated by Ficoll density-gradient centrifugation. Recovered cells were washed twice with phosphate-buffered saline and once in growth medium consisting of Medium 199 (GIBCO BRL Life Technologies) supplemented with 20 percent fetal-calf serum, penicillin (100 U per milliliter), and streptomycin (100 μg per milliliter). Isolated cells were subsequently resuspended in growth medium and plated on dishes coated with human fibronectin (Biocoat, Becton Dickinson Labware). To eliminate the possibility of contaminating the assay with mature circulating endothelial cells, we performed an initial preplating step in a fibronectin-coated six-well plate using 5 million peripheral-blood mononuclear cells per well. After 48 hours, the nonadherent cells were collected and 1 million cells were replated onto fibronectin-coated 24-well plates for a final assess-

ment of the number of colonies. Growth medium was changed every three days, and the numbers of colonies were counted seven days after plating.

A colony of endothelial progenitor cells consisted of multiple thin, flat cells emanating from a central cluster of rounded cells. A central cluster alone without associated emerging cells was not counted as a colony. Colonies were counted manually in a minimum of four wells by observers who were unaware of the subjects' clinical profiles. Confirmation of endothelial-cell lineage was performed in samples from 10 subjects as previously described.[1,24] Briefly, indirect immunostaining was performed with the use of endothelial-specific antibodies directed against vascular endothelial growth factor receptor 2 and CD31 or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate–acetylated low-density lipoprotein and costaining with BS-1 lectin.

To assess reproducibility, we determined the colony counts twice in two separate blood samples obtained at least one week apart from 10 subjects. The samples were analyzed independently by two observers who were unaware of the subjects' clinical profiles. The interobserver correlation was 0.92, whereas the intraclass correlation, obtained by a single observer who analyzed two blood samples obtained at least one week apart from a single subject, was 0.97.

For the measurement of cellular senescence, we recruited on the basis of the Framingham score a subgroup of 16 age-matched subjects from the original 45 subjects. The subgroup was then divided into a high-risk group and a low-risk group, with eight in each group (mean scores, 7.3±2.3 and 1.5±2.1, respectively; P<0.001). Cultures of endothelial progenitor cells from these subjects were maintained for seven days, and the medium was changed every three days. Senescence-associated β-galactosidase activity was measured as previously described.[25] Isolated cells distant from central colonies were analyzed, and only cells with a distinctly blue cytoplasm, indicating β-galactosidase activity, were counted. The percentage of positive cells was determined by counting four random fields, which contained a total of approximately 100 to 200 cells.

ASSESSMENT OF ENDOTHELIUM-DEPENDENT AND ENDOTHELIUM-INDEPENDENT FUNCTION

Brachial reactivity was assessed in the morning after an overnight fast. Imaging of the brachial artery proximal to the antecubital fossa was performed with the use of high-resolution ultrasonography (12.5-MHz linear-array transducer, model ATL HDI 5000, Advanced Technology Laboratories), as previously reported.[26,27] Endothelium-dependent flow-mediated vasodilatation (flow-mediated brachial reactivity) was assessed by measuring the maximal increase in the diameter of the brachial artery during reactive hyperemia evoked by the release of a cuff inflated to 225 mm Hg for five minutes on the upper arm, proximal to the measurement site. After a rest period of 15 minutes, base-line measurements (diameter and flow velocity) were repeated, and 0.4 mg of nitroglycerin spray was administered sublingually to assess endothelium-independent vasodilatation.

Before the subjects were enrolled in this study, we conducted an eight-week study of the reproducibility of the entire procedure of flow-mediated and nitroglycerin-induced brachial reactivity using a single observer and seven subjects. Measurements of the diameter of the brachial artery at rest (3.77 mm initially and 3.72 mm on repeated measurement, r=0.99), during flow-mediated dilatation (4.02 and 4.0 mm, respectively; r=0.97), and after the administration of nitroglycerin (4.23 and 4.09 mm, respectively; r=0.88) were reproducible. The magnitude of flow-mediated vasodilatation was similar at base line and at eight weeks (12.7±0.8 percent and 11.9±0.8 percent, respectively; P=0.70). Furthermore, the interobserver variability of the ultrasonographic analysis (performed twice in blinded fashion by a single operator) had a correlation coefficient of 0.99.

STATISTICAL ANALYSIS

Data are expressed as means ±SE. The means for subjects in the high-cardiovascular-risk group were compared with those in the low-risk group with the use of a two-tailed unpaired Student's t-test. The chi-square test was used for comparisons of categorical variables. Univariate correlations were performed with use of Spearman's correlation coefficient. Results were verified with use of the nonparametric Wilcoxon rank-sum test. To identify predictors of changes in colony counts of endothelial progenitor cells in a multivariate setting, we used multiple linear regression (General Linear Model Procedure, SAS) on specific variables. A similar analysis was conducted with respect to determinants of flow-mediated brachial reactivity.

RESULTS

FORMATION OF ENDOTHELIAL-PROGENITOR-CELL COLONIES AND CARDIOVASCULAR RISK FACTORS

Peripheral-blood mononuclear cells formed distinct colonies on fibronectin-coated dishes (Fig. 1). We and other investigators have previously demonstrated that endothelial progenitor cells isolated in this fashion exhibit many endothelial characteristics, including expression of CD31, TIE2, and vascular endothelial growth factor receptor 2.[1,24] We next assessed whether the level of circulating endothelial progenitor cells correlated with the presence or absence of conventional cardiovascular risk factors. The numbers of endothelial-progenitor-cell colony-forming units were significantly reduced in subjects with elevated serum cholesterol levels ($P=0.002$), hypertension ($P=0.04$), and diabetes ($P=0.04$). We also observed an inverse correlation between the subject's age and levels of circulating endothelial progenitor cells; however, this relation was not statistically significant. When, in this small group of relatively healthy subjects, the individual risk factors of cholesterol levels, hypertension, and diabetes were also adjusted for age, only hypercholesterolemia remained significant ($P=0.004$). To determine whether the cumulative risk was associated with endothelial-progenitor-cell counts, we calculated the Framingham risk score for each subject and found a significant inverse correlation between the score and endothelial-progenitor-cell counts ($r=-0.47$, $P=0.001$), with higher scores associated with diminished counts (Fig. 2).

COUNTS OF ENDOTHELIAL-PROGENITOR-CELL COLONIES AND ENDOTHELIUM-DEPENDENT AND ENDOTHELIUM-INDEPENDENT FUNCTION

We next assessed the relation between endothelial-progenitor-cell colony counts and flow-mediated brachial reactivity, a composite measure of endothelial integrity. As shown in Figure 3, there was a strong correlation between the colony count and flow-mediated brachial reactivity ($r=0.59$, $P<0.001$). When the flow-mediated brachial reactivity was divided into three subgroups, subjects with the highest level of reactivity had colony counts that were approximately three times as high as those with the lowest level (mean, 24.5 ± 3.6 vs. 7.8 ± 1.5 colony-forming units; $P<0.001$). We also observed a correlation between the number of endothelial progenitor cells and the response to nitroglycerin, an endothelium-independent stimulus ($r=0.40$, $P=0.007$). To
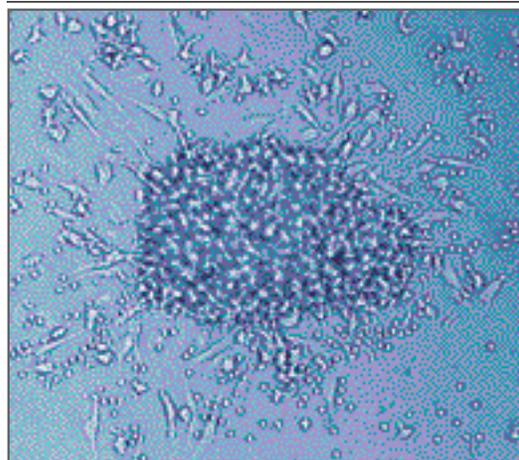

Figure 1. Phase-Contrast Micrograph of an Endothelial-Progenitor-Cell Colony Characterized by a Central Cluster of Rounded Cells Surrounded by Radiating Thin, Flat Cells (×200).
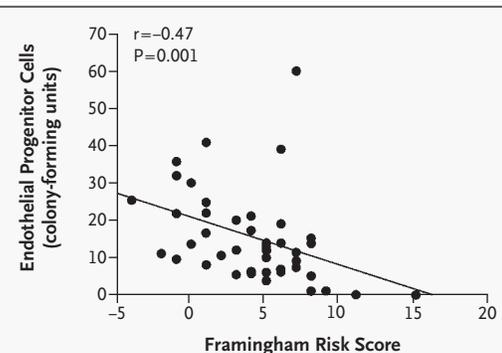

Figure 2. Association between Cardiovascular Risk Factors and Endothelial-Progenitor-Cell Colony Counts.

The number of colony-forming units was strongly correlated with the subjects' Framingham risk score. Levels of endothelial progenitor cells were expressed as the mean number of colonies per well in at least four separate determinations for each subject. Higher scores on the Framingham risk score indicate greater cardiovascular risk.

understand whether the relation between flow-mediated brachial reactivity and cell counts was independent of vascular smooth-muscle function, we determined the ratio of flow-mediated brachial reactivity to nitroglycerin responsiveness for each subject. Again, subjects with the highest ratio of flow-mediated brachial reactivity to nitroglycerin had
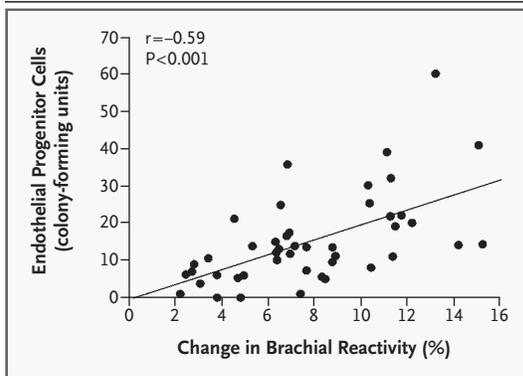
Figure 3. Relation between the Number of Endothelial Progenitor Cells and Endothelial Function.

Flow-mediated brachial reactivity was expressed as the percent change from base line after the release of an occlusive cuff.

higher cell counts than did subjects with the lowest ratio (20.4±3.8 vs. 8.1±1.2, P=0.01).

Finally, multivariate regression analysis was performed to determine whether the number of endothelial-progenitor-cell colonies was associated with age, race, body-mass index, cigarette smoking, hypertension, diabetes, total cholesterol levels, glucose levels, brachial flow-mediated brachial reactivity, or responses to nitroglycerin. This analysis demonstrated that flow-mediated brachial reactivity was an independent predictor of the number of endothelial-progenitor-cell colonies (P<0.001). A reciprocal analysis that divided subjects into three groups according to endothelial-progenitor-cell activity also demonstrated a striking relation between the level of endothelial progenitor cells and flow-mediated brachial reactivity (Table 1).

Table 1. Characteristics of the 45 Patients According to the Level of Circulating Endothelial Progenitor Cells.*

Characteristic	All Subjects (N=45)	High Cell Count, 28.4±3.0 (N=15)	Intermediate Cell Count, 12.4±0.4 (N=15)	Low Cell Count, 4.7±0.8 (N=15)	P Value†
Age — yr	50±2	46±3	50±3	55±3	0.07
Body-mass index	28±0.6	28±1.0	27±1.0	28±1.0	0.80
Glucose — mg/dl	100±5.0	92±3.0	101±11.0	107±8.0	0.09
Total cholesterol — mg/dl	200±6.0	182±11.0	193±11.0	226±7.0	0.002
Low-density lipoprotein cholesterol — mg/dl	138±5.0	127±9.0	131±8.0	157±7.0	0.02
High-density lipoprotein cholesterol — mg/dl	48±2.0	49±3.0	46±2.0	50±3.0	0.80
Triglycerides — mg/dl	148±16	112±16	150±27	181±36	0.09
Insulin — μU/ml	16.1±3.0	12±2	21±8	15±3	0.46
Hypertension — no. (%)	10 (22)	1 (7)	1 (7)	8 (53)	0.01
Diabetes — no. (%)	10 (22)	0	5 (33)	5 (33)	0.04
Smoker — no. (%)	3 (7)	1 (7)	0	2 (13)	1.00
Framingham risk score‡	4.2±0.6	1.8±0.8	4.1±0.8	6.6±0.9	<0.001
Flow-mediated brachial reactivity — % change from base line	7.8±0.5	10.0±0.8	8.2±0.8	5.2±0.7	<0.001
Nitroglycerin response — %	12.6±0.6	14.3±1.0	12.4±0.9	11.3±1.0	0.06

* Plus–minus values are means ±SE. Body-mass index is the weight in kilograms divided by the square of the height in meters. To convert values for glucose to millimoles per liter, multiply by 0.05551. To convert values for cholesterol to millimoles per liter, multiply by 0.02586. To convert values for triglycerides to millimoles per liter, multiply by 0.01129.

† P values are from a t-test comparison of the highest and lowest cell-count groups. Noncategorical results were verified with the use of nonparametric tests and were adjusted for age. All statistically significant relations remained significant in subsequent analyses.

‡ The Framingham risk score can range from –6 to 19, with higher scores indicating greater cardiovascular risk.

ENDOTHELIAL PROGENITOR CELLS AND FLOW-MEDIATED BRACHIAL REACTIVITY

We next divided subjects into four approximately equal subgroups on the basis of their Framingham risk scores and numbers of endothelial-progenitor-cell colonies. As shown in Figure 4, the subjects with high cell counts (greater than 13; mean, 23) had preserved flow-mediated brachial reactivity irrespective of whether they had a high or low risk score. Similarly, those with low cell counts (13 or fewer colonies; mean, 7) had depressed flow-mediated brachial reactivity, independently of whether their risk score was high or low. From these observations, it would appear that the activity of endothelial progenitor cells is a better predictor of endothelial function than the presence or absence of conventional risk factors. Indeed, when assessed alone, the Framingham risk score was significantly correlated with flow-mediated brachial reactivity ($P=0.016$). However, in a multivariate analysis of flow-mediated brachial reactivity that included both the Framingham risk score and the number of endothelial progenitor cells as variables, the cumulative risk score lost its significance ($P=0.27$), whereas the endothelial-progenitor-cell counts were significant

Figure 4. Correlation of the Mean (+SE) Activity of Endothelial Progenitor Cells with Flow-Mediated Brachial Reactivity.

The 45 subjects were divided into four approximately equal subgroups on the basis of the endothelial-progenitor-cell counts (expressed as colony-forming units) and the Framingham risk score. The activity of endothelial progenitor cells was a stronger predictor of flow-mediated brachial reactivity than the presence or absence of conventional cardiovascular risk factors. Flow-mediated brachial reactivity was expressed as the percent change from base line.

($P=0.003$) over and above the effects of the Framingham risk score.

CARDIOVASCULAR RISK AND SENESCENCE OF ENDOTHELIAL PROGENITOR CELLS

If endothelial progenitor cells from high-risk subjects undergo use-dependent depletion, we speculated that the cells remaining in circulation might demonstrate in vitro characteristics of clonal exhaustion, accelerated aging, or both. To assess this possibility further, we measured endogenous cellular β-galactosidase activity, a marker of cellular senescence, in a subgroup of 16 subjects selected because they had either high or low cumulative Framingham risk scores (7.3 ± 2.3 and 1.5 ± 2.1, respectively; $P<0.001$) but similar chronologic ages (mean age, 49.1 ± 5.9 and 54.6 ± 9.3 years, respectively; $P=0.85$). After seven days in culture, there was a significant difference in the percentages of endothelial progenitor cells with a senescent phenotype: 27 ± 9 percent of the cells derived from the low-risk subjects and 72 ± 15 percent of the cells from the high-risk subjects had β-galactosidase staining ($P=0.005$).

DISCUSSION

Endothelial damage ultimately represents a balance between the magnitude of injury and the capacity for repair. A variety of evidence suggests that cardiovascular risk factors induce endothelial injury and that impaired endothelial function reflects this ongoing injury. Little is known about the mechanisms by which the vessel wall undergoes repair. We postulated that circulating endothelial progenitor cells constitute one aspect of this repair process.

Low levels of circulating endothelial progenitor cells in patients with increasing cardiovascular risk could be a byproduct of a number of mechanisms. Presumably, risk factors, by modulating the levels of oxidative stress, nitric oxide activity, or other physiologic processes, could directly influence the mobilization or half-life of endothelial progenitor cells. Consistent with this explanation are observations demonstrating that the initiation of statin therapy increases the levels of circulating endothelial progenitor cells.[28-30] An alternative explanation that we explored is that continuous endothelial damage or dysfunction leads to an eventual depletion or exhaustion of a presumed finite supply of endothelial progenitor cells. This process is analogous to what has been observed in patients with
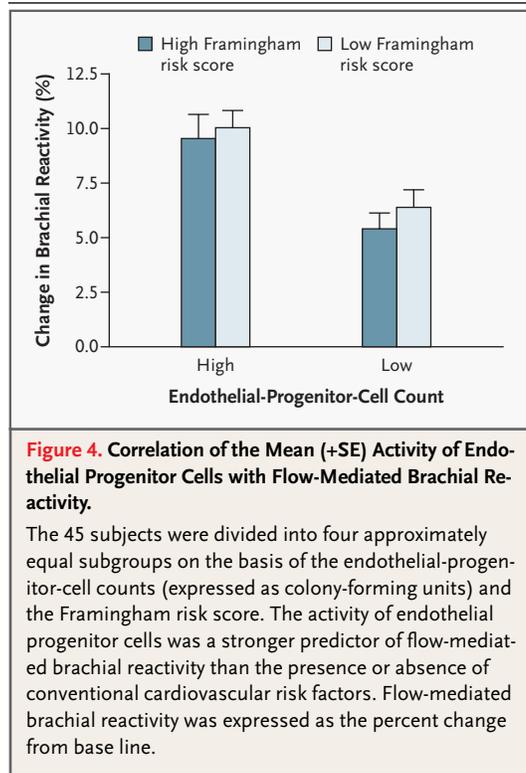
muscular dystrophy. Owing to the continuous cycles of damage and repair associated with the underlying diathesis, patients with dystrophic muscle eventually exhaust their supply of resident progenitor cells, a type that is termed "satellite cells" in skeletal muscle. In addition, the few satellite cells that remain within the muscle bed have evidence of accelerated aging.[31-33] This observation may be analogous to the correlation we found between the presence or absence of progenitor cells and the maintenance or impairment of endothelial function. In addition, we found that endothelial progenitor cells from high-risk subjects are both fewer in number and become senescent more rapidly than those from low-risk subjects. Similarly, previous studies have noted other qualitative differences between endothelial progenitor cells from patients with symptomatic coronary artery disease and those from control subjects.[34]

The nature and size of our study do not permit us to determine whether low levels of endothelial progenitor cells can accurately predict subsequent cardiovascular events. Similarly, we cannot deduce from our observations that a decrease in endothelial progenitor cells impairs flow-mediated brachial reactivity. Establishing a definitive cause-and-effect relation requires studies in which the levels of endothelial progenitor cells are experimentally manipulated and the biologic or therapeutic effects assessed. Rather, we believe our data suggest that circulating endothelial progenitor cells have a role in vascular homeostasis. We further speculate, but cannot prove, that continuous risk-factor–induced injury may lead to the eventual depletion of circulating endothelial progenitor cells. Interestingly, recent studies in animals have suggested that the exhaustion of stem cells may be an important determinant of a number of age-related conditions.[35,36] Future studies will therefore be needed to determine whether this postulated risk-factor–induced exhaustion of circulating endothelial progenitor cells is a factor in the pathogenesis of cardiovascular disease.

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