Influence of BMI on Level of Circulating Progenitor Cells

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Obesity complicates a number of diseases through mechanisms that are poorly defined. Mobilization and recruitment of progenitor cells to pathological sites is an important factor in disease progression. Here, we analyzed the influence of obesity on the systemic circulation of CD34+ cell populations and correlated frequencies of cells displaying previously established cell marker signatures with the BMI. Comparative analysis of peripheral blood mononuclear cells (PBMC) from 12 nonobese (BMI <30 kg/m^2) and 14 obese (BMI >30 kg/m^2) disease-free donors by flow cytometry revealed that obesity is associated with a fivefold increased frequency of circulating progenitor cells (CPC), a population consisting of hematopoietic and endothelial precursors. Our data also indicate that obesity is associated with increased frequency of circulating mesenchymal stromal progenitor cells (MSC). In contrast, the frequencies of mature endothelial cells (EC) and CD34-bright leukocytes are unaffected by obesity. Combined, our results indicate that obesity promotes mobilization of progenitor cells, which may have clinical relevance.

Obesity, a wide-spreading medical condition, is associated with a range of life-threatening diseases including type-2 diabetes, cardiovascular disease, and cancer through mechanisms that are poorly understood (1–3). It has become clear that white adipose tissue (WAT), overgrown in obesity, is the source of factors that have systemic effects on many aspects of physiology. Mobilization of progenitor cells and their recruitment to the site of inflammation is one of the mechanisms that underlies tissue repair and influences disease progression (4). Elevated systemic circulation of hematopoietic progenitor cells (HPC), endothelial progenitor cells (EPC), and of stromal progenitors, commonly referred to as mesenchymal stromal cells (MSC) underlies tissue remodeling in development and pathology (5,6). Although the capacity of progenitor cells to facilitate wound healing can have clinical benefit, it can also negatively affect disease outcome. For example, recruitment of HPC, EPC, and MSC by tumors and fibrotic lesions can promote cancer progression through effects on vascularization, stromatogenesis, and the immune response (3,7,8). The purpose of this study was to examine the relationship between obesity and levels of circulating progenitor cells (CPCs).

SUBJECTS AND METHODS

In this study, 26 individuals (11 men and 15 women) with the mean age of 45 ± 3 years were recruited. Approval for the study was obtained from Tulane University institutional review board. Peripheral blood samples (15 ml) were collected from each subject under an informed consent.

Demographic (age, gender), medical (history of diabetes, cancer and other comorbidities), and lifestyle (smoking, exercise) data were collected, BMI (kg/m^2) was calculated, and subjects were divided into subgroups according to their BMI (nonobese <30 and obese >30). **Supplementary Table S1** online shows the baseline donor characteristics.

For flow cytometric analysis of human blood, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. PBMC analysis was performed with an LSR-II flow cytometer and the FACSDiva software (BD Bioscience, San Jose, CA). Cells were gated to exclude cell clumps, contaminating polymorphonuclear cells, red blood cells, platelets, endothelial microparticles, debris, as well as dead cells based on 7-aminoactinomycin D staining (**Figure 1a**). Viable PBMC (>500,000/sample) were then used to enumerate individual populations (**Figure 1b**). For fluorescence-activated cell sorting on PBMC and WAT-derived cells (see **Supplementary Figure S1** online), fluorescein isothiocyanate-conjugated CD31 antibody (clone WM59), phycoerythrin-conjugated CD34 antibody (clone 8G12), and allophycocyanin-Cy7-conjugated CD45 antibody (clone HI30) along with appropriate isotype control immunoglobulin G from BD Bioscience were used. Cell culturing, cytospins, cell differentiation assays, and cell staining analyses were performed as we have described previously (7,9,10).

Statistical comparisons of circulating cell frequencies, which were not normally distributed as determined by the Kolmogorov–Smirnov test, were performed using nonparametric Mann–Whitney U-test (for two group comparison) and by using ANOVA based on the Kruskal–Wallis test (for three group comparison).

RESULTS

To analyze systemic circulation of CD34+ cells, we enumerated distinct peripheral blood cell populations by multiparametric

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flow cytometry. Based on the established gating protocol (11), we enumerated circulating CD34dimCD31brightCD45⁻ endothelial cells (EC) and CD34brightCD31dimCD45dim CPC, which include HPC and EPC (11). In parallel, we quantified circulating CD34brightCD45bright leukocytes and cells with the CD34brightCD31⁻CD45⁻ immunophenotype; the latter were present in the majority of PBMC from obese subjects (**Figure 1b**). We used conventional cell culture assays (7,9,10) to establish the identity of each cell population isolated by fluorescence-activated cell sorting (**Figure 1c**). Cells sorted as CPC predominantly did not attach in culture and had the expected HSC morphology. A shown in **Supplementary Figure S2a,b** online, we demonstrated that this population is indeed enriched in hematopoietic progenitors based on their capacity to differentiate into unit-granulocyte-macrophage or burst-forming units-erythrocyte. As expected, the other three sorted cell populations were not efficient in hematopoiesis (see **Supplementary Figure S2a,b** online). Cells sorted as CD34bright leukocytes expectedly contained nonadherent and weakly adherent cells, whereas cells sorted as EC attached to fibronectin and attempted forming endothelial networks. The identity of EC was validated by immunofluorescence analysis of cytospins indicating the expected expression of von Willebrand factor and VE-Cadherin (CD144), which also confirmed lack of endothelial markers on other sorted populations (see **Supplementary Figure S2c** online). The CD34brightCD45⁻CD31⁻ population has not been previously identified in blood by flow cytometry. In solid tissues, the CD34brightCD45⁻CD31⁻ immunophenotype has been previously attributed to pericytic mesenchymal progenitors (MSC) that can be isolated as fibroblast colony-forming units (CFU-F) (10,12,13). To test whether these cells correspond to circulating MSC, we analyzed cytospins of every cell population isolated by fluorescence-activated cell sorting by immunofluorescence. This confirmed expression of NG2, a pericyte marker present on MSC, specifically by CD34brightCD45⁻CD31⁻ cells (see **Supplementary Figure S2c** online). We also inspected PBMC cultured on uncoated plastic in conditions used for MSC expansion (7,12). Indeed, we observed CFU-F typical of MSC derived from PBMC of subjects with BMI >30 (**Figure 1d**). Interestingly, we have not been able to isolate CFU-F from PBMC of any of the BMI <30 subjects. We confirmed that cultured cells of this population express characteristic MSC markers such as collagen-I and αSMA (see **Supplementary Figure S3a** online). Differentiation of culture-expanded CFU-F from obese individuals into adipocytes, osteoblasts, and chondrocytes by using standard assays established in our group (13) unequivocally demonstrated their MSC identity (see **Supplementary Figure S3b** online).

Finally, we assessed whether obesity correlates with changes in cell circulation in healthy volunteers assigned into two groups based on the BMI: nonobese (BMI <30), and obese (BMI >30). Fifty-four percent of the study subjects had BMI >30. There were no significant differences between the BMI groups with regard to mean age, gender, physical activity, or dieting history (see **Supplementary Table S1** online).

Circulating cell frequency data for all of the individuals analyzed are presented in **Supplementary Table S2** online and graphically summarized in **Figure 1e**. Consistent with previously reported results (11,14), PBMC obtained from nonobese donors contained 0.02% CPC and 0.76% EC (**Figure 1e**). In this group, CD34bright leukocytes constituted 0.03% of viable PBMC, whereas MSC were at the background level of detection (0.001%). Assignment of nonobese subjects into lean (BMI <25) and overweight (BMI 25–30) revealed no significant difference in cell circulation between these subgroups (see **Supplementary Figure S4** online). By comparing BMI <30 and BMI >30 PBMC, we detected no statistically significant correlation between obesity and frequencies of circulating EC ($P = 0.3681$) or CD34bright leukocytes ($P = 0.2268$). In stark contrast, obese subjects displayed a fivefold higher ($P = 0.0019$) frequency of circulating CPC and a tenfold higher ($P = 0.0021$) frequency of circulating MSC, as compared to nonobese subjects (**Figure 1e**).

DISCUSSION

Here, we enumerated circulating CPC, EC, CD34bright leukocytes, and MSC by flow cytometry and confirmed the identity of these populations through phenotypic characterization *ex vivo*. Although the strategy for enumeration of hematopoietic and endothelial populations was based on previously published studies (11,14), this is the first report on MSC enumeration by flow cytometry. We show that flow cytometric separation of EC, CPC, and CD34-bright leukocytes isolates the corresponding cell populations from both PBMC (**Figure 1**) and from the stromal/vascular fraction of WAT (see **Supplementary Figure S1** online). As reported previously (10,15,16), our gating strategy also isolates MSC as the CD34brightCD45⁻CD31⁻ population from WAT. Our inability to directly recover CFU-F from the small numbers of cells sorted as MSC from PBMC is likely due to their comparatively large size and the resulting fragility/vulnerability to damage during sorting. However, expression of pericyte/MSC markers specifically on circulating CD34brightCD45⁻CD31⁻ cells combined with the presence of multipotent CFU-F exclusively in the peripheral blood of obese individuals strongly argues that these cells are indeed MSC.

Previously, increased circulation of white blood cells in obese individuals has been reported (17). So far, it has not been established which cell populations are mobilized resulting in increased white blood cells counts of obese subjects. Our data demonstrate that obesity is associated with increased circulation of hematopoietic and mesenchymal progenitor cells. Previous studies have reported increased circulation of HSC, EPC, and EC under pathological conditions such as cancer (6,8,14). Although association of HPC circulation with obesity has not been previously explored, a recent study reported an inverse correlation of EPC circulation with obesity (18). Because EPC constitute only a minor fraction of the combined CPC pool, it can be concluded that mobilization of HPC is responsible for the association of obesity with increased frequency of CPC that we observed. Our results indicate that MSC circulate at detectable levels in obese, but not in lean or overweight individuals. This observation is consistent

with published reports on MSC detection in the systemic circulation, which have been until now based on CFU-F enumeration in cell culture (5). In agreement with our inability to detect MSC in nonobese donors, CFU-F circulation has been previously found virtually undetectable in healthy individuals and shown to be increased in certain pathological conditions (19). Strikingly,

according to previous reports, the "MSC-positive" blood samples contain CFU-F at a frequency of $1-2/10^6$ white blood cells (20), which is in the range of the MSC content we established for the majority of PBMC samples from nonobese individuals.

Because WAT expanded in obesity is a rich source of progenitor cells (15,16), our findings suggest a possibility that at

Figure 1 Enumeration of cell populations in peripheral blood. (**a**) FSC-H/FSC-A graph: gating on single cells; exclusion of cell clumps, erythrocytes, platelets, endothelial cells (EC) microparticles, and debris. SSC-A/FSC-A graph: gating on mononuclear cells. 7-AAD/SSC-A graph: gating on viable peripheral blood mononuclear cells (PBMCs). The gating diagram on the right indicates that circulating progenitor cells (CPC), CD34bright (CD34b) leukocytes (LC), EC, and CD34bCD45−CD31− cells are enumerated as sub-populations of viable PBMC. (**b**) Enumeration of circulating cells by flow cytometry: representative data from a lean (upper panel) and an obese (lower panel) donor. The cutoffs for CD31, CD34, and CD45 bright, dim, and negative (−) cells, as indicated, are based on the position of previously characterized (11) CPC and EC populations; CD34bCD45−CD31− cells and CD34b LC are identified relative to EC and CPC localization on the plots. The plots on the right include only cells gated as CPC and CD34bCD45−CD31− cells in the middle panels. (**c**) Phase contrast micrographs of cell cultures (1 week) derived from the corresponding cell populations sorted in **b**. Note the expected morphology of HPC, EC, and monocytoid CD34b leukocytes attached to plastic. (**d**) A clone of mesenchymal stromal cells (MSC) derived from PBMC of a BMI >30 subject after 3 weeks of culture. Bar = 50 μM. (**e**) Frequencies of cell populations in PBMC as a function of BMI. Flow cytometric enumeration of CPC, EC, CD34b LC, and CD34bCD45−CD31− cells was performed for disease-free donors with BMI <30 (*N* = 12), and BMI >30 (*N* = 14). Shown are mean % of viable PBMC ± s.e. of the mean. *$P < 0.01$ (Mann–Whitney *U*-test).

least some of MSC and CPC observed in the peripheral blood of obese subjects originate in adipose tissue. In addition to adipocytes, WAT contains high numbers of MSC (known as adipose stromal cells), as well as EC and infiltrating white blood cells (10,21), which is demonstrated in **Supplementary Figure S1** online. Recently, WAT has also been appreciated as an ectopic reservoir of HPC (22). In concert with adipocytes, these WAT cell populations secrete endocrine molecules collectively termed adipokines (23). While the evidence for bioactivity of WAT-derived progenitors is burgeoning, their mobilization, migration, and implication in human disease so far have not been explored. In mouse models, adipose EC and MSC can migrate to sites of chronic inflammation and contribute to tumor microenvironment (7). Recent studies indicating that MSC are quickly recruited to the sites of tissue damage from the circulation (24) are consistent with our unpublished data from patients undergoing open abdominal surgery (data not shown). The possibility of mobilization of WAT cells and their recruitment by pathological tissues in humans remains to be further investigated. On the other hand, recent reports indicating that adipocytes can be derived from hematopoietic stem cells (25–27) point to the possibility of "hematopoietic to mesenchymal transition." Although these reports have been challenged with data indicating that bone marrow-derived precursors contribute only to infiltrating vasculature-associated cells, but not to adipocytes (28,29), increased MSC circulation in obese subjects due to cell mobilization from the bone marrow, rather than from WAT, remains a possibility. Although the exact molecular networks driving migration of cells in disease and obesity are unknown at present, it is likely that inflammation and hypoxia signals are important (30). Irrespective of the source of cells mobilized in obesity, our results may have important clinical implications. If progenitors mobilized in obesity become recruited to the sites of disease, they may positively influence tissue repair and recovery, thus possibly accounting for the "obesity paradox" (31).

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/oby

DISCLOSURE
The authors declared no conflict of interest.

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