BRIEF REPORT

Adipose Mesenchymal Stromal Cells Isolated From Type 2 Diabetic Patients Display Reduced Fibrinolytic Activity

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Stem cells have been successfully used for the treatment of critical limb ischemia (CLI). We conducted a clinical trial to determine the feasibility of using autologous adipose-derived mesenchymal stromal cells (AdMSCs) for the treatment of CLI. Unexpectedly, two diabetic patients developed peripheral microthrombosis. This adverse effect, which contrasts with the reported antithrombotic properties of MSCs, may stem from the diabetic environment that alters the fibrinolytic activity of AdMSCs, thereby increasing the probability of developing thrombosis. Here, we confirm this premise by demonstrating that diabetic AdMSCs cultured in the presence of blood sera expressed and released higher levels of plasminogen activator inhibitor type 1, reduced levels of tissue plasminogen activator, and lower D-dimer formation compared with nondiabetic AdMSCs. Thus, to establish an appropriate cell therapy for diabetic patients, we recommend including new preclinical safety tests, such as the D-dimer and/or the tissue plasminogen activator-to-plasminogen activator inhibitor type 1 ratio tests, to assess fibrinolytic activity of cells before implantation. *Diabetes* 62:4266–4269, 2013

W e evaluated the safety and feasibility of using autologous adipose-derived mesenchymal stromal cells (AdMSCs) for diabetic patients (ClinicalTrials.gov identifier: NCT01257776). Two diabetic patients developed distal microthrombosis (DMT) that was controlled with aggressive antithrombotic therapy. DMT was not detected when autologous AdMSCs isolated from nondiabetic patients were used (ClinicalTrials.gov identifier: NCT01745744) under identical conditions, as reported in Table 1, or when autologous bone marrow mononuclear cells (BMMNCs) were transplanted in diabetic patients with critical limb ischemia (CLI) (ClinicalTrials.gov identifier: NCT00872326) (1). Development of AdMSCs-associated DMT has not been previously reported and is in sharp contrast to the described fibrinolytic and antithrombogenic properties of MSCs (2–4). This novel clinical observation raises the possibility that the diabetic milieu of patients may alter the functional properties of AdMSCs, thereby impairing secretion of factors involved in fibrinolysis. To validate this premise, we evaluated whether AdMSCs isolated from diabetic and nondiabetic patients and cultured in the presence of healthy, diabetic, or nondiabetic blood sera displayed differential expression levels of tissue plasminogen activator (tPA) and plasminogen activator inhibitor type 1 (PAI-1), which may result in impaired fibrinolytic activity of AdMSCs derived from diabetic patients.

RESEARCH DESIGN AND METHODS

Blood samples. Human serum was from diabetic (hBS-D) and nondiabetic (hBS-ND) patients with CLI, as well as from healthy volunteers (hBS). All donors provided informed consent that was formerly approved by local and regional medical research ethics committees.

AdMSCs procurement. AdMSCs were isolated from abdominal adipose tissue biopsy specimens of diabetic (AdMSC-D) and nondiabetic (AdMSC-ND) patients diagnosed with CLI and enrolled in two independent clinical trials (EudraCT: 2008-001387-88 and EudraCT: 2009-013554-32), as previously described (5). AdMSCs were cultured in Mesenchymal Stem Cell Basal Medium (MSCBM, Lonza, Barcelona, Spain) complemented with growth supplements (GS: MSCGM SingleQuots, Lonza), 100 units/mL penicillin-streptomycin, and incubated at 37°C in a 20% O_2 and 5% CO_2 humidified atmosphere. Control AdMSCs were purchased from ATCC.

Experimental setup. AdMSCs, AdMSC-D, and AdMSC-ND were plated at a density of 5×10^4 cells/cm². The following day, the media was replaced with serum/GS-free MSCBM (Lonza) and 24 h after with MSCBM (Lonza) supplemented with GS, hBS, hBS-D, or hBS-ND. Supernatants were collected for ELISA analysis and cells harvested for RNA extraction 24 h after sera addition.

Quantitative RT-PCR. cDNA was generated from 2 μg RNA using M-MLV Reverse Transcriptase (Promega Biotech, Alcobendas, Spain). Primer sequences for tPA and PAI-1 were designed using Primer3 Software (http://frodo.wi.mit.edu/primer3/) and can be obtained upon request, whereas the housekeeping gene primers for cyclophilin A (PPIA) and 60S acidic ribosomal protein P0 (RPLP) were purchased from Tataabiocenter (Göteborg, Sweden). QT-PCR was performed using a SensiFAST SYBR Lo-ROX Mix (Bioline Reagents Ltd) and a 7500 Real-Time PCR System (Applied Biosystems). Three distinct amplifications were performed for each transcript, and the C_T assay was applied to assess relative transcript levels of tPA and PAI-1.

tPA and PAI-1 secretion assay. Levels of total tPA (free and complexed) and total PAI-1 (active complexed with tPA and latent inactive) secreted in the media of the various experimental groups were quantified using precoated ELISA plates (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions.

Fibrin gel culture assay. Fibrin gels were prepared as previously described (6). Subsequent to polymerization, 1 mL MSCBM without serum or with GS, hBS, hBS-D, or hBS-ND was added to the clotted fibrin gels. Medium was collected 24 h later for D-dimer detection using the Blue D-Dimer Kit (Teco, Neufahrn, Germany). Gels were fixed in formalin and processed for hematoxylin-eosin staining. The free-cell/area was quantified from four arbitrary images per slide per condition using ImageJ freeware.

Statistical analysis. Unless otherwise stated, data are represented as mean values ± SEM. Data were analyzed with one-way and two-way ANOVA tests. The Tukey test was used for comparisons. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

We initially assessed the relative transcript levels of the fibrinolytic promoting factor tPA as well as the amount of

TABLE 1
Clinical parameters and inclusion and exclusion criteria of diabetic and nondiabetic patients with CLI

Inclusion criteria	Exclusion criteria
Written informed consent	Malignancy and hematological
Age	Cardiac disease risk factors
$\geq$18 and $\leq$75 years old (patients with diabetes)	Severe heart failure (NYHA IV)
$\geq$18 and $\leq$89 years old (nondiabetic individuals)	Malignant ventricular arrhythmias or unstable angina
Both sexes	Stroke or myocardial infarction within the last 3 months
Population target	Ejection fraction of the left ventricle less than 30%
Diabetes type 1 or 2 (HbA$_{1c}$ $\geq$6.5%; fasting blood	Uncontrolled hypertension
glucose 4–7 mmol/L)	Biochemical parameters
Ischemic nondiabetic patients	Anemia (hemoglobin <7.9 mg/dL)
Infrapopliteal atherosclerotic vascular disease	Leukopenia
of severe grade with severe claudication or	Thrombocytopenia (<100,000 platelets/μL)
Rutherford-Becker grade II–III or IV	Deep vein thrombosis in the previous 3 months
Ankle-brachial pressure index at rest <0.8	Untreated proliferative retinopathy
Failed attempts of surgical or endovascular	HIV, hepatitis B or hepatitis C virus
revascularization (PTA/stents or bypass)	Other risk factors
Biochemical parameters	BMI >40 kg/m^2
Leukocytes $\geq$3,000/μL	Predicted impossibility to obtain a biopsy
Neutrophils $\geq$1,500/μL	specimen providing 10 g adipose tissue
Platelets $\geq$100,000/μL	Patients who participated in a clinical trial within
AST/ALT $\leq$2.5 units/L	the last 3 months before inclusion in this clinical trial
Creatinine $\leq$2.5 mg/dL	Alcoholism
Women of childbearing potential must have negative	
results on a pregnancy test	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; NYHA, New York Heart Association; PTA, percutaneous transluminal angioplasty. A total of 60 patients with CLI (30 diabetic patients and 30 nondiabetic patients) fulfilled the same inclusion and exclusion criteria and enrolled in the following clinical trials: ClinicalTrials.gov identifiers NCT01257776 (diabetic) and NCT01745744 (nondiabetic).

secreted protein in isolated AdMSCs, -D, and -ND cultured in the presence of GS (control), hBS, -D, and -ND. In the control condition, all three cell populations exhibited similar tPA mRNA expression levels. However, when cultured in media supplemented with blood sera (hBS, -D, and -ND), tPA transcript levels and secreted protein levels were significantly lower in AdMSC-D compared with AdMSCs and AdMSC-ND, suggesting that the addition of sera, independent of its origin, inhibited tPA expression in AdMSC-D (Fig. 1A and B).

We next evaluated whether levels of PAI-1 were also altered in AdMSC-D compared with AdMSCs and AdMSC-ND. Overexpression of PAI-1 has been shown to potentially compromise normal fibrin clearance mechanisms and promote pathological fibrin deposition and thrombotic events (7,8). PAI-1 transcript levels were consistently higher in AdMSC-D than in AdMSC-ND in all culture conditions (Fig. 1C). Interestingly, AdMSCs exhibited a similar PAI-1 expression pattern as AdMSC-D. Nonetheless, at the protein levels, AdMSC-D secreted the highest quantity of PAI-1, reaching maximum levels in the presence of hBS-D (400 ng/mL), whereas AdMSCs and AdMSC-ND, independent of serum condition, released ~150 to 200 ng/mL protein. In presence of GS (control), AdMSCs and AdMSC-D both secreted similar low levels, whereas AdMSC-ND released barely detectable amounts of PAI-1 (Fig. 1D).

We next measured the fibrinolytic activity of the various AdMSCs. Cells were embedded into fibrin clots to mimic an ex vivo fibrinolytic situation and cultured with GS or in the presence of blood sera (hBS, -D, and -ND). Fibrin degradation, as judged by a steady clearing zone surrounding single cells, was significantly hampered in AdMSC-D compared with AdMSCs or AdMSC-ND. This effect appeared to be independent of serum conditions (Fig. 1E and F). In contrast, AdMSCs diplayed the highest number of cell free/area, reaching maximum levels in the presence of hBS-ND. Interestingly, AdMSC-ND cultured in the presence of hBS-D produced significantly less cell free/area than cells cultured in hSB or hSB-ND (Fig. 1E and F). To validate this observation, we measured levels of D-dimer, a by-product of fibrin degradation (Fig. 1G). Consistent with lower cell free/area, AdMSC-D displayed blunted capacity to generate D-dimers, independent of the serum (Fig. 1G). Interestingly, D-dimer production from AdMSC-ND cultured in hBS-D was as low as from AdMSC-D, suggesting that blood derived from diabetic patients may also contain other factors that inhibit fibrinolysis without altering tPA and PAI-1 levels (Fig. 1B and D).

DISCUSSION

Our results clearly demonstrate that diabetic-derived AdMSCs have an altered phenotype indicating the existence of a potential post-transcriptional mechanism that regulates PAI-1 expression levels, typified by its increase resulting in blunted fibrinolytic activity. Exposing AdMSC-D to hBS or hBS-ND did not alter this phenotype, indicating that long-term fibrinolytic modifications have potentially been endowed that cannot be reverted under normal culture conditions. The latter may therefore impart increased susceptibility to develop DMT in patients who receive transplants of diabetic-derived AdMSCs. Interestingly, AdMSC-ND cultured in hBS-D exhibited decreased fibrinolytic activity even though tPA and PAI-1 transcript levels and secreted proteins were not significantly altered by sera conditions. These data suggest that blood-derived factors associated with hyperglycemia most likely also contribute to decrease fibrinolytic activity. To our knowledge, this is the first case report on the adverse effect of using diabetic-derived AdMSCs for cell therapy, which should be taken into account
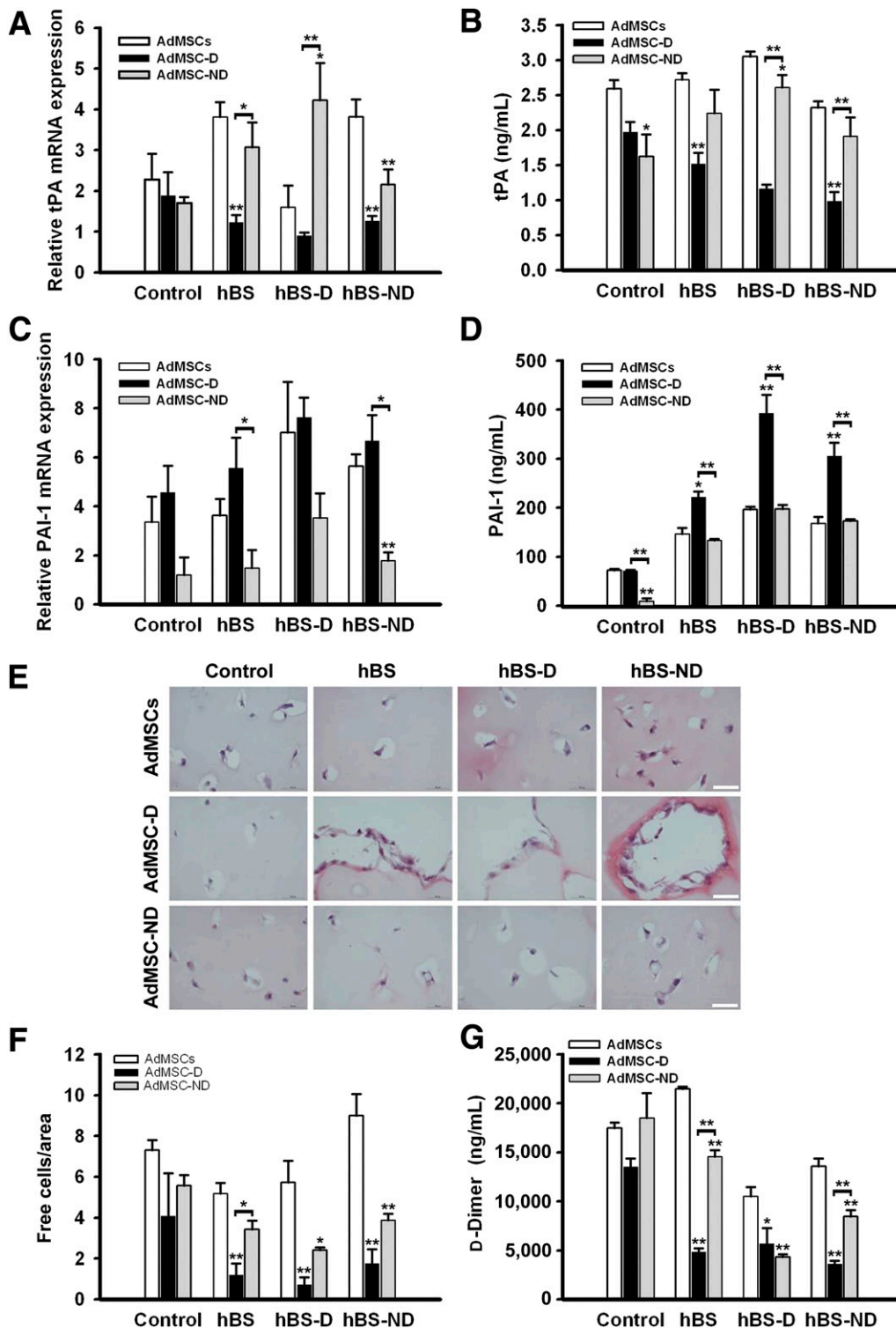
FIG. 1. AdMSCs derived from diabetic patients display reduced and serum-independent fibrinolytic activity. Expression analysis of tPA and PAI-1 I shown in AdMSCs isolated from AdMSCs, AdMSC-D, and AdMSC-ND and cultured in the presence of GS (control), hBS, hBS-D, and hBS-ND. Detection of tPA mRNA expression (*A*). tPA protein release shows that the blood sera, independent of its origin, inhibited tPA expression in AdMSC-D (*B*). PAI-1 mRNA expression (*C*). *D*: PAI-1 protein release. Note that AdMSC-D secreted the highest quantity of PAI-1. Relative mRNA expression indicates the ratio between specific gene and housekeeping genes. Values are normalized to the expression in no stimulated AdMSCs, which was arbitrarily set as 1. *E*: Hematoxylin and eosin staining shows AdMSCs-mediated lysis of the fibrin gel. The cell is surrounded by a clearing zone, except for AdMSC-D, which displays hampered capacity to degrade fibrin gel independently of human blood serum used. Scale bars: 50 µm. *F*: Free cell/area quantification from hematoxylin-eosin staining of arbitrary images. *G*: In quantification of D-dimer production of AdMSCs cultured in fibrin clot, AdMSC-D displayed blunted capacity to generate D-dimers independently of serum used. Data are presented as mean values ± SEM (*n* = 4). Statistical significance: *$P \leq 0.05$, **$P \leq 0.01$ for AdMSC-D compared with AdMSC-ND.

for future clinical management. Thus, because MSCs are widely used in cell therapy, we would recommend including new preclinical safety tests, such as the D-dimer and/or tPA–to–PAI-1 ratio tests, to assess fibrinolytic activity of cells before implantation. Such testing will aid in establishing an appropriate therapy after implantation therapy tailor-made for each individual patients.

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