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Gap Junction–Mediated Transport of Metabolites Between Stem Cells and Vascular Endothelial Cells

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Abstract

We have previously demonstrated that small molecular transfer, such as glucose, between hematopoietic stem cells (HSCs) or mesenchymal stem cells (MSCs) and vascular endothelial cells via gap junctions constitutes an important mechanism of stem cell therapy. Cell metabolites are high-potential small-molecule candidates that can be transferred to small molecules between stem cells and vascular endothelial cells. Here, we investigated the differences in metabolite levels between stem cells (HSCs and MSCs), vascular endothelial cells, and the levels of circulating non-hematopoietic white blood cells (WBCs). The results showed remarkable differences in metabolite concentrations between cells. Significantly higher concentrations of adenosine triphosphate (ATP), guanosine triphosphate (GTP), total adenylate or guanylate levels, glycolytic intermediates, and amino acids were found in HSCs compared with vascular endothelial cells. In contrast, there was no significant difference in the metabolism of MSCs and vascular endothelial cells. From the results of this study, it became clear that HSCs and MSCs differ in their metabolites. That is, metabolites that transfer between stem cells and vascular endothelial cells differ between HSCs and MSCs. HSCs may donate various metabolites, several glycolytic and tricarboxylic acid cycle metabolites, and amino acids to damaged vascular endothelial cells as energy sources and activate the energy metabolism of vascular endothelial cells. In contrast, MSCs and vascular endothelial cells regulate each other under normal conditions. As the existing MSCs cannot ameliorate the dysregulation during insult, exogenous MSCs administered by cell therapy may help restore normal metabolic function in the vascular endothelial cells by taking up excess energy sources from the lumens of blood vessels. Results of this study suggested that the appropriate timing of cell therapy is different between HSCs and MSCs.

Keywords

hematopoietic stem cells, mesenchymal stem cells, human umbilical vein endothelial cells, circulating CD34-negative and CD45-positive white blood cells, gap junction

Introduction

With the recent development of stem cell research, cell therapy has been studied as a new therapeutic strategy for various diseases[1–13]. Hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are major cell sources in cell-based therapies. However, the therapeutic mechanism of each cell therapy has not been fully elucidated, which is attributed to the complex composition of cell therapy. We demonstrated previously that transplanted HSCs increase vascular endothelial growth factor (VEGF) uptake into vascular endothelial cells via gap junction–mediated cell–cell interaction and the transfer of small molecules, such as glucose, from HSCs to endothelial cells via gap junction as an energy source triggers angiogenesis[14]. In contrast, we also demonstrated transplanted MSCs decrease VEGF uptake into vascular endothelial cells through gap junction–mediated cell–cell interaction and suppress inflammation[15]. For both HSCs and MSCs, cell–cell interactions via gap junctions comprise a key mechanism for their cell therapy.

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However, the VEGF-mediated effects of HSCs and MSCs on endothelial cells were opposing.

The gap junction channel is composed of two hemichannels, named connexons, which connect the cytoplasm of two cells directly[16]. Each connexon comprises six subunit proteins called connexins (Cx). An individual connexon from one cell docks or associates with a corresponding connexon on a neighboring cell to form a gap junction channel and multiple channels. Multiple channels cluster or aggregate in the plane of the membrane to form gap junction plaques[17–19]. The properties of gap junction channels are defined by the Cx. Vascular endothelial cells are mutually regulated not only between allogeneic cells with adjacent vascular endothelial cells but also between heterologous cells such as smooth muscle cells, pericytes, and blood cells via gap junction[14,15]. Gap junctions allow the intercellular exchange of regulatory ions or small molecules (maximum mass of 1.5 kDa) between adjacent cells and are the only channels mediating direct cytoplasmic exchange, a process known as gap junction intercellular communication[19–22]. Small molecules can be transported freely between cells via gap junctions depending on the concentration gradient in the cytoplasm of the cells. However, the selectivity or priority of the molecules that actually pass through the gap junctions has not been clarified. Cell–cell communication and signaling are regulated by the exchange of small soluble molecules between cells through gap junction channels[13]. This exchange between stem cells and vascular endothelial cells via gap junctions is important for both HSCs and MSCs in cell therapies after stroke[14,15]. In our previous study, we focused on glucose, one of the small soluble molecules being transported from HSCs to endothelial cells and from endothelial cells to MSCs, via gap junction channels as an energy source[14,15]. However, currently, there is insufficient knowledge on other substances, other than glucose, transported via gap junction channels between HSCs, MSCs, and endothelial cells.

This study aims to examine the cellular metabolism of HSCs, MSCs, vascular endothelial cells, and non-hematopoietic white blood cells (WBCs) and identify the small soluble molecules transported between these cells along concentration gradients. All cells used in this study are known to interact with vascular endothelial cells. Here, we defined HSCs as CD34-positive ($CD34^+$) cells, non-hematopoietic WBCs as $CD34^-/CD45^+$ cells, and human bone marrow–derived MSCs as MSCs and compare the metabolism of these cells with that of human umbilical vein vascular endothelial cells (HUVECs).

Materials and Methods

The following study using human cord blood cells was approved by the Ethics Committee of the Foundation for Biomedical Research and Innovation at Kobe.

Preparation of Human HSC ($CD34^+$ Cells)

In this study, we defined HSC as CD34-positive cells. Human cord blood from six donors was purchased by the Japan Red Cross. Mononuclear cells were isolated by Ficoll-Paque (GE Healthcare, Little Chalfont, UK) density-gradient centrifugation as described previously[23,24]. $CD34^+$ cells were isolated using a Direct CD34 Progenitor Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocols.

Preparation of Human Non-Hematopoietic WBCs ($CD34^-/CD45^+$ Cells)

We defined non-hematopoietic WBCs as $CD34^-/CD45^+$ cells, in this report. $CD34^-$ mononuclear cells were collected as non-$CD34^+$ cells from the cord blood from six human donors and isolated using CD45 microbeads according to the manufacturer's protocol (Miltenyi Biotec, 130-045-801).

Preparation of Human MSCs

Human bone marrow–derived MSCs were purchased from Lonza (Basel, Switzerland) from six healthy different donors. MSCs were cultured with growth medium (PT3001: Lonza) according to the manufacturer's protocol. Cells in passage 4 were used for this experiment.

Preparation of Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were cultured from Kurabo (Osaka, Japan) for six different donors (healthy male, neonate, Caucasian, or African American) with medium, serum, and growth factors (HuMedia-EB2; Kurabo) according to the manufacturer's protocol. After reaching 80%–90% confluence, cells were dissociated with 0.025% Trypsin-EDTA (Kurabo) and expanded. HUVECs in passage 6 were used for all experiments.

Metabolome Analysis

Metabolic extracts were prepared from 1×10^6 cells of $CD34^-/CD45^+$, $CD34^+$ cells, MSCs, and HUVECs. Culture medium was removed from the dish, and cells were washed twice in 5% mannitol solution (10 ml first and then 2 ml). Cells were then treated with 800 µl of methanol and 550 µl of Milli-Q water containing the internal standard solution [H3304-1002; Human Metabolome Technologies (HMT), Inc., Tsuruoka, Yamagata, Japan]. The metabolite extract was transferred into a 1.5-ml tube and centrifuged at $2,300 \times g$ and 4°C for 5 min. Next, the upper aqueous layer was

centrifugally filtered through a Millipore 5-kDa cutoff filter at 9,100 × *g* and 4°C for 120 min to remove proteins. All samples were stored at −80°C until measurement.

Metabolome analysis was performed at HMT[25–27]. The filtrate was centrifugally concentrated and resuspended in 50 ml of Milli-Q water for capillary electrophoresis–mass spectrometry (CE-MS) analysis. Cationic compounds were analyzed in the positive mode of capillary electrophoresis–time-of-flight mass spectrometry (CE-TOFMS) using the Agilent CE-TOFMS system (Agilent Technologies, Santa Clara, CA, USA). Cationic metabolites were analyzed with a fused-silica capillary (50 μm i.d., 80 cm total length) with cation buffer solution (HMT) as the electrolyte. The samples were injected at a pressure of 50 mbar for 5 s. The applied CE voltage was set at 30 kV. Electrospray ionization mass spectrometry (ESI-MS) was conducted in positive ion mode, and the capillary voltage was set at 4,000 V. The spectrometer was scanned from *m/z* 50 to 1,000. Anionic metabolites were analyzed with a fused-silica capillary (50 mm i.d., 80 cm total length) with anion buffer solution (HMT) as the electrolyte. The samples were injected at a pressure of 50 mbar for 5 s. The applied voltage was set at 30 kV. ESI-MS was conducted in positive and negative ion mode, and the capillary voltage was set at 4,000 V for positive and 3,500 V for negative mode. To obtain peak information, *m/z* ratio, migration time, and peak area detected by CE-TOFMS and CE-MS/MS were extracted using automatic integration software (MasterHands ver.2.17.1.11, Keio University, Tsuruoka, Japan and MassHunter Quantitative Analysis B.04.00, Agilent Technologies). The tolerance range for the peak annotation was configured at ±0.5 min for migration time and ±10 ppm for *m/z*. In addition, concentrations of metabolites were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and by using standard curves, which were obtained by three-point calibrations.

Animals

This article adheres to the American Heart Association Journals Implementation of the Transparency and Openness Promotion Guidelines. All animal experiments were approved by the Animal Care and Use Committee of Foundation for Biomedical Research and Innovation and complies with the Guide for the Care and Use of Animals published by the Japanese Ministry of Education, Culture, Sports, Science and Technology. All efforts were made to minimize the suffering and number of animals used in this study.

Data Analysis

For all experiments in this report, mean ± SEM are reported ($n = 6$). Statistical comparisons among groups were determined using a Kruskal–Wallis test followed by Dunn's test, with the statistical significance set at $P < 0.05$.

Results

Metabolome Analysis

Metabolome analysis was performed on four types of cells: $CD34^+$ HSCs, non-hematopoietic WBCs ($CD34^-/CD45^+$), vascular endothelial cells (HUVECs), and human bone marrow MSCs. The measurement targets included the glycolytic substrates, tricarboxylic acid (TCA) cycle, purine metabolism, the nicotinamide metabolism pathway, the pentose phosphate pathway, glutathione metabolism, and various amino acids.

Adenylate and guanylate energy charge. Cells generate ATP, the major currency for energy-consuming reactions, through central carbon metabolism, which includes glycolysis and mitochondrial oxidative phosphorylation. The adenylate or guanylate energy charge is an index used to measure the energy status of biological cells. The highest concentrations of ATP or GTP and total adenylate or total guanylate levels were observed in $CD34^+$ cells, which are HSCs, and total adenylate or total guanylate levels were also the highest in $CD34^+$ cells (Fig. 1A, B). In contrast, $CD34^-/CD45^+$ cells differentiated from HSCs showed significantly lower levels of both adenylate energy charge and guanylate energy charge than $CD34^+$ cells or MSCs.

Glycolysis and TCA cycle. Glycolysis is the first step in the production of energy. In glycolysis, sugar molecules are phosphorylated and trapped in the cell to be catabolized into two pyruvate molecules, which are the end products of glycolysis. The highest concentrations of glycolytic substrates were observed in $CD34^+$ HSCs. However, the ratios of dihydroxyacetone phosphate (DHAP)/glycerol 3-phosphate (G3P) and lactate/pyruvate were significantly higher in HUVECs (Fig. 2). Among the TCA cycle intermediates, the highest concentration of citric acid was found in $CD34^-/CD45^+$ cells.

Amino acids. As shown in Fig. 3, $CD34^+$ HSCs had the highest concentrations of all amino acids, except Cys and Trp. The $CD34^-/CD45^+$ cells showed significantly lower amino acid concentrations than $CD34^+$ cells. In addition, HUVECs and MSCs were found to contain similar amounts of all amino acids, except Cys and Pro. Total essential amino acids included a total of 9 essential amino acids (His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val), and a total of 11 non-essential amino acids (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr). In both cases, $CD34^-/CD45^+$ cells exhibited the lowest number and $CD34^+$ cells the highest. The concentrations of the total essential and non-essential amino acids were comparable in HUVECs and MSCs. Similar trends were observed for both the total amounts of amino acids metabolized to pyruvate or acetyl CoA, intermediates of glycolytic pathway, and the total amounts of amino acids metabolized to fumaric acid, an intermediate TCA cycle.

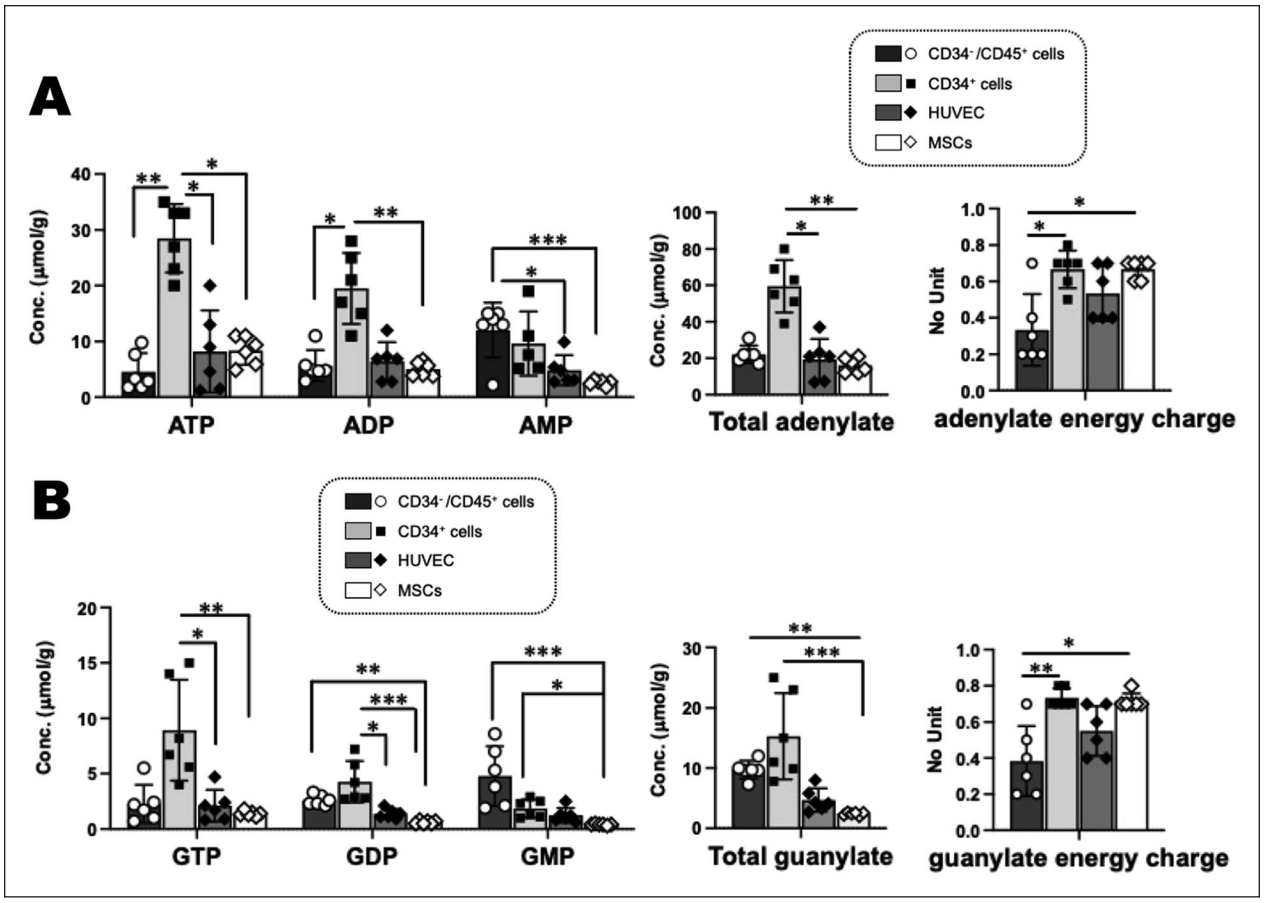

Figure 1. (A) Total adenylate and adenylate energy charge; (B) total guanylate and guanylate energy charge in four cell types. Metabolite levels in four cell types, namely, $CD34^-/CD45^+$ cells, $CD34^+$ cells, HUVECs, and MSCs, were determined using a capillary electrophoresis–time-of-flight mass spectrometer. Results were expressed as mean ± standard error of mean (SEM) ($n = 6$). ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; GTP: guanosine triphosphate; GDP: guanosine diphosphate; HUVECs: human umbilical vein vascular endothelial cells; MSCs: mesenchymal stem cells.
* indicates statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Nicotinamide metabolism. The nicotinamide adenine dinucleotide (NADH) cofactor is essential for a variety of cellular processes. Owing to its reducing activity, nicotinamide adenine dinucleotide phosphate (NADPH) plays an important role in protecting cells from oxidative stress by its reducing action[28]. The ratios of $NADH/NAD^+$ or $NADPH/NADP^+$ determine the intracellular redox potential. The $NADPH/NADP^+$ or $NADH/NAD^+$ ratios were the highest in $CD34^-/CD45^+$ cells (Fig. 4A).

De novo *synthesis of purine nucleotides.* There are three pathways of purine metabolism: degradation to uric acid, *de novo* synthesis, and the salvage pathway. *De novo* purine synthesis represents a basis for all other steps in nucleotide metabolism. The levels of phosphoribosyl pyrophosphate (PRPP) and inosinic acid (IMP), which are involved in *de novo* purine synthesis, were significantly higher in $CD34^-/CD45^+$ cells than in HUVECs or MSCs (Fig. 4B). The degradation products of IMP, inosine and hypoxanthine, were significantly higher in HUVECs.

Pentose phosphate pathway. The pentose phosphate pathway is involved in the *de novo* synthesis of purine nucleotides. Moreover, the conversion of $NADP^+$ to NADPH is mainly catalyzed by the pentose phosphate pathway, with high $NADPH/NADP^+$ ratios indicating pentose phosphate pathway activation. We observed that the pentose phosphate pathway was activated in $CD34^+$ and $CD34^-/CD45^+$ cells (Fig. 5).

Glutathione metabolism. Glutathione (GSH) is the most abundant antioxidant in aerobic cells. GSH is critical for protecting the brain from oxidative stress, acting as a free radical scavenger and inhibitor of lipid peroxidation. GSH also participates in the detoxification of hydrogen peroxide by various glutathione peroxidases. The ratio of reduced GSH to oxidized GSH [glutathione disulfide (GSSG)] is an indicator of cellular health, with a decrease in this ratio suggesting the progression of oxidative stress. For the four cell types studied here, the total glutathione level tended to be the highest in CD34, whereas the GSH/GSSG ratio was the highest in HUVECs and was lower in $CD34^-/CD45^+$ cells and MSCs (Fig. 6).

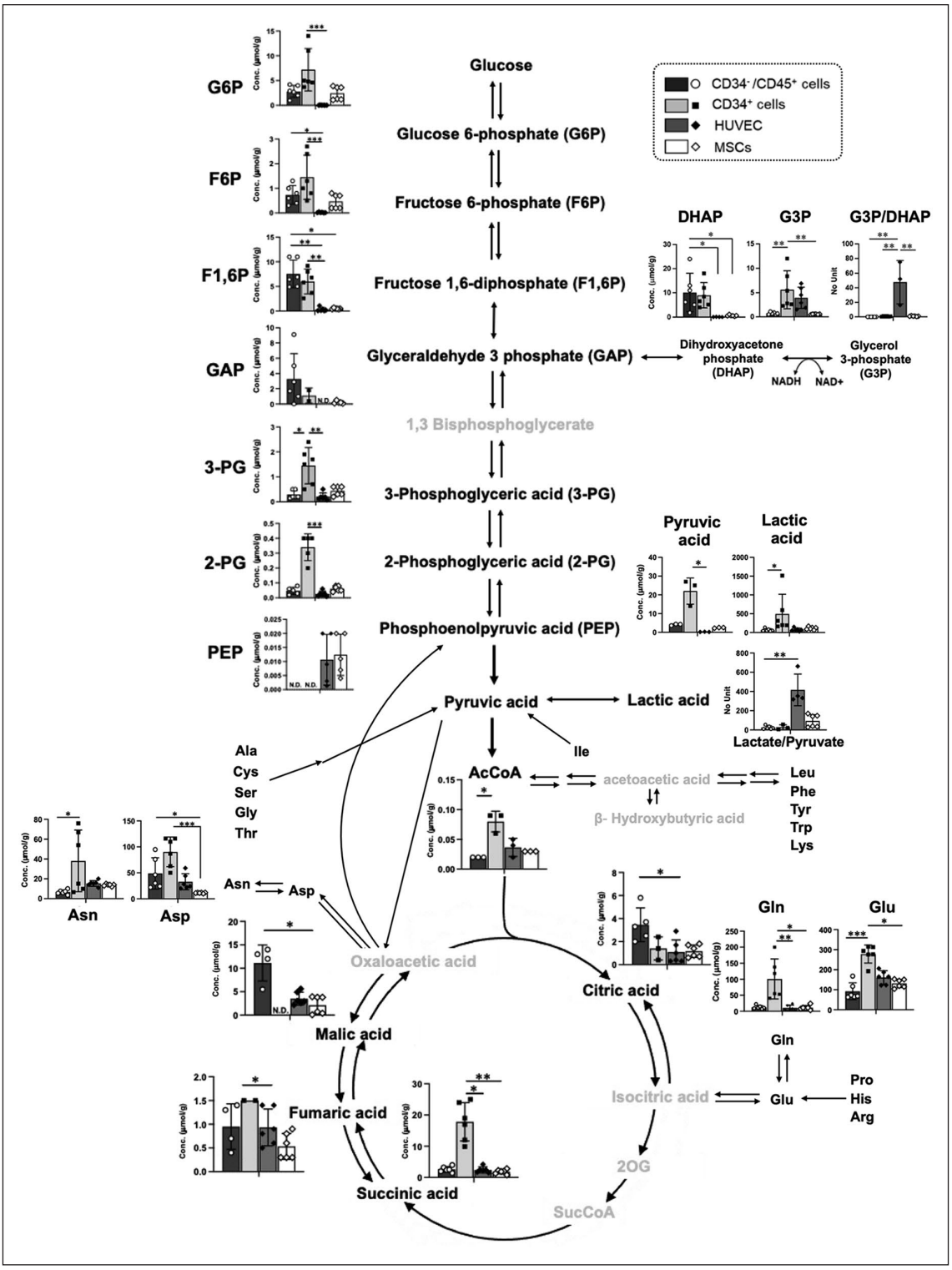

Figure 2. Central metabolic pathway map and comparative visualization of involved metabolites. CD34$^+$ HSCs showed the highest levels of glycolytic intermediates among the four cell groups. Black font indicates detected substances, and gray font indicates not detected in any of the four cell types. Results were expressed as ean ± standard error of mean (SEM) ($n = 6$). Black font indicates detected substances and gray font indicates not detected in all four cell types. AcCoA: Acetyl-CoA; ADP: adenosine diphosphate; Ala: alanine; AMP: adenosine monophosphate; Arg: arginine; Asn: asparagine; Asp: aspartic acid; ATP: alanine triphosphate; Cys: cystein; GDP: guanosine diphosphate; Gln: glutamine; Glu: glutamic acid; GTP: guanosine triphosphate; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; N.D.: not detected; NAD$^+$/NADH: nicotinamide adenine dinucleotide; Phe: phenylalanine; Pro: proline; Ser: serine; Thr: threonine; Trp: tryptophan; Tyr: tyrosine.
* indicates statistical significance: *$P < 0.05$; **$P < 0.01$;***$P < 0.001$.

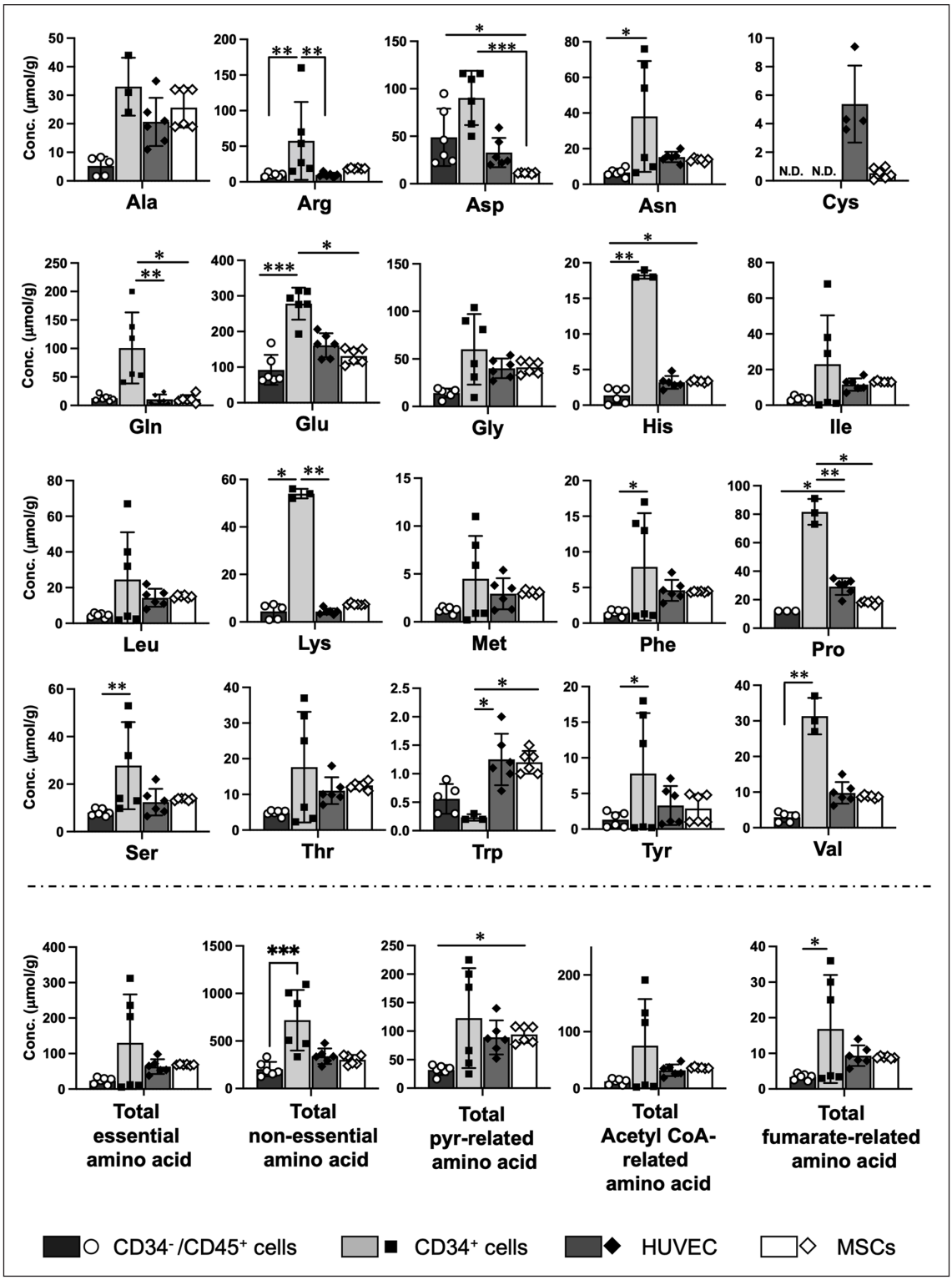

Figure 3. Amino acid concentrations in four types of cells. The results of the four-group comparison showed that levels of most of the amino acids were significantly higher in HSCs. Results were expressed as mean ± standard error of mean (SEM) (n = 6). Ala: alanine; Arg: arginine; Asn: asparagine: Asp: aspartic acid; Cys: cystein; Gln: glutamine; Glu: glutamic acid; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; Phe: phenylalanine; Pro: proline; Ser: serine; Thr: threonine; Trp: tryptophan; Tyr: tyrosine; Val: valine; HSCs: hematopoietic stem cells.

* indicates statistical significance: *$P < 0.05$; **$P < 0.01$;***$P < 0.001$.

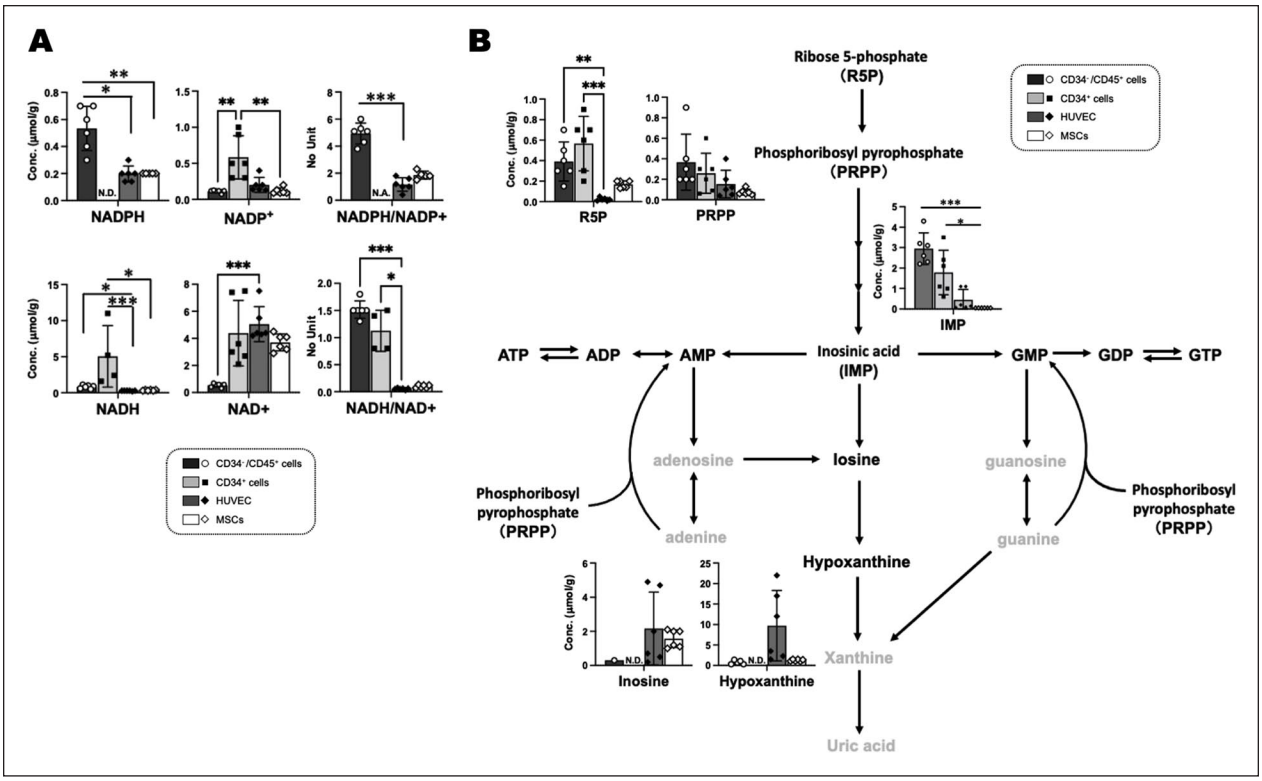

Figure 4. Nicotinamide metabolism and *de novo* synthesis of purine nucleotides. (A) The ratios of NADH/NAD$^+$ or NADPH/NADP$^+$ were the highest in CD34$^-$/CD45$^+$ cells. (B) PRPP and IMP, which are involved in the *de novo* synthesis of purine nucleotides, were significantly higher in CD34$^-$/CD45$^+$ cells than in HUVECs or MSCs. The degradation products of IMP—inosine and hypoxanthine—were significantly higher in HUVECs. Results were expressed as mean ± standard error of mean (SEM) ($n = 6$). Black font indicates detected substances and gray font indicates not detected in all four cell types. N.A.: not available; N.D.: not detected; PRPP: phosphoribosyl pyrophosphate; IMP: inosinic acid; HUVECs: human umbilical vein vascular endothelial cells; MSCs: mesenchymal stem cells.
*indicates statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

In Vivo *Transfer of a Low-Molecular-Weight Fluorescent Substance From Mice Circulating WBCs to Human Endothelial Cells*

We had previously reported cell–cell interactions between HSCs and vascular endothelial cells, MSCs and vascular endothelial cells, and MSCs and circulating WBCs[14,15]. In the present study, we examined the interaction between circulating WBCs and vascular endothelial cells. 2′-7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) is a low-molecular-weight fluorescent compound known to pass through gap junctions[29]. To evaluate the *in vitro* interaction between circulating WBCs and HUVECs, BCECF was loaded into the mouse circulating WBCs, co-cultured with HUVECs, and the BCECF signal in HUVECs was evaluated by fluorescence-activated cell sorting. In contrast to the low fluorescence intensity of HUVECs following co-culture without cells, a prominent BCECF signal was observed in HUVECs after co-culture with BCECF-loaded mouse circulating WBCs (Supplemental Figure). This result indicated that circulating WBCs and vascular endothelial cells also exchange molecules through cell–cell interactions.

Human VEGF Uptake into HUVECs

VEGF is one of the most prominent proangiogenic factors[30]. HUVECs are known to secrete VEGF into culture medium and uptake it in an autocrine/paracrine manner[31]. In our previous study, we demonstrated that mouse bone marrow mononuclear cells (mBM-MNCs), which contain some HSCs and have a higher glucose concentration, supply glucose to human vascular endothelial cells via gap junctions, followed by increased uptake of VEGF into endothelial cells, leading to the activation of angiogenesis[14]. Then, we examined the effect of mouse WBCs on the uptake of VEGF in HUVECs. WBCs did not promote the uptake of VEGF, despite interacting with vascular endothelial cells (Supplemental Table).

Discussion

This study analyzes the metabolomes of HSCs, circulating non-hematopoietic WBCs (CD34$^-$/CD45$^+$), vascular endothelial cells (HUVECs), and MSCs. The results showed that HSCs are in a high-energy state with an activated glycolytic system, and the differentiation of HSCs into peripheral

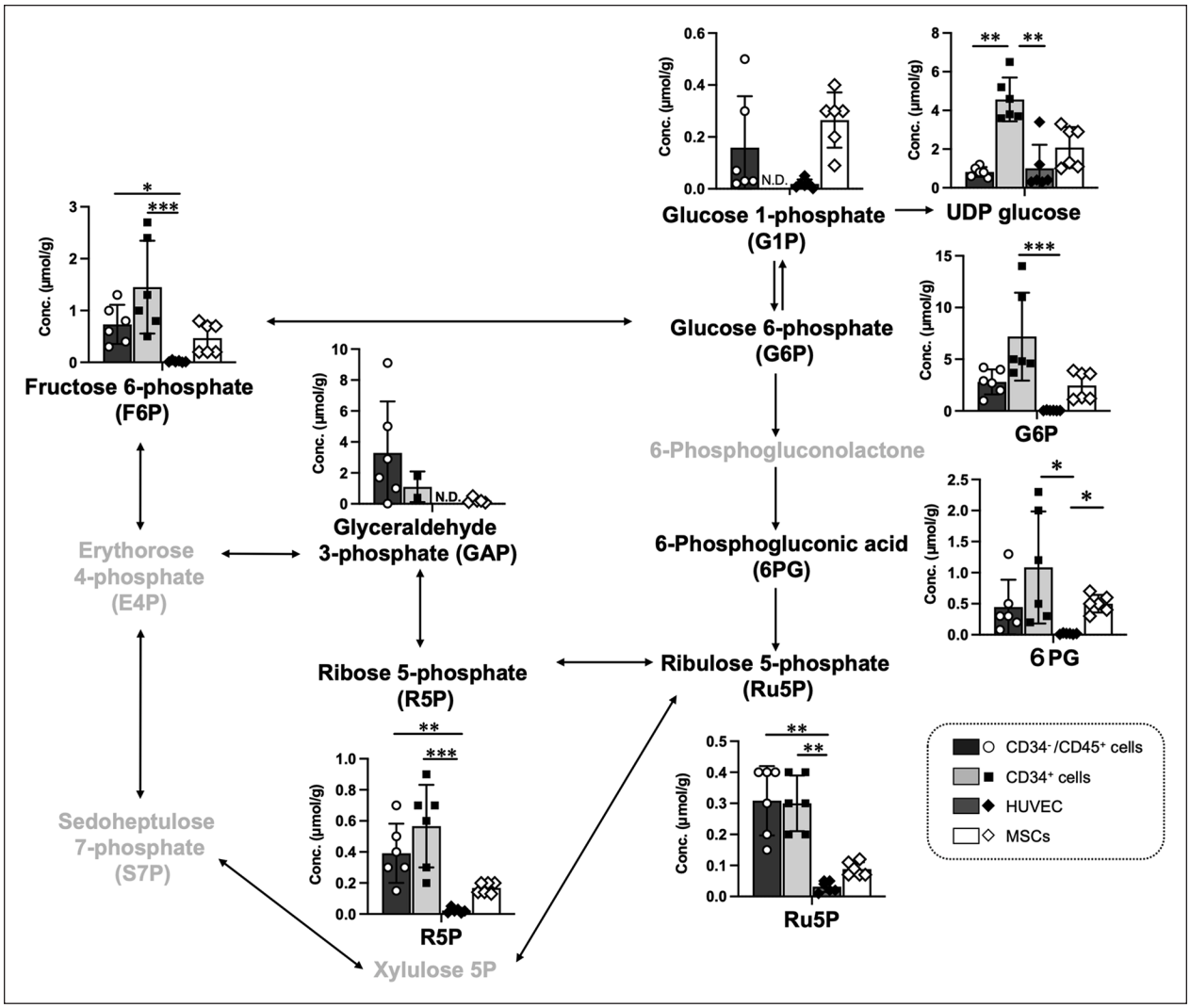

Figure 5. Pentose phosphate pathway in four types of cells. The pentose phosphate pathway was upregulated in $CD34^+$ and $CD34^-/CD45^+$ cells. Results were expressed as mean ± standard error of mean (SEM) (n = 6). N.A.: not available; N.D.: not detected; UDP: uridine diphosphate.
*indicates statistical significance: *$P < 0.05$; **$P < 0.01$;***$P < 0.001$.

blood leukocytes results in an energy-consuming tendency. In contrast, MSCs showed almost the same metabolic tendency as normal vascular endothelial cells—HUVECs in this study.

HSCs, which contained fewer mitochondria than progenitors[32], use anaerobic metabolism in the hypoxic endosteal zone[28,33]. Although the ratio of ATP generation to glucose consumption via anaerobic glycolysis is inefficient compared with that supported by oxidative phosphorylation, the rate of ATP production under hypoxia potentially increases 100-fold compared with that supported by mitochondrial energy production under normoxic conditions. The results of this study showed that HSCs have a significantly higher concentration of glycolytic substrate, indicating that the glycolytic system is activated in HSCs. The amino acid concentrations are also higher in HSCs. Hematopoietic stem cell therapy has had a significant therapeutic effect on ischemic stroke[34]. Branched-chain amino acids are reduced in ischemic stroke, and the degree of reduction correlates with poorer neurological outcome[35]. Therefore, it is likely that the supply of amino acids from HSCs contributes to the activation of energy metabolism in vascular endothelial cells.

Gap junction channels allow the intracellular exchange of small molecules, such as ions and metabolites, according to their concentration gradient between cells. We had previously shown that the glucose concentrations in HSCs were significantly higher than in HUVECs and that glucose was transported from the HSCs into the HUVECs through gap junctions[14]. Taken together, these results suggest that it is highly likely that many molecules, in addition to glucose, may be transferred through gap junctions. Thus, the

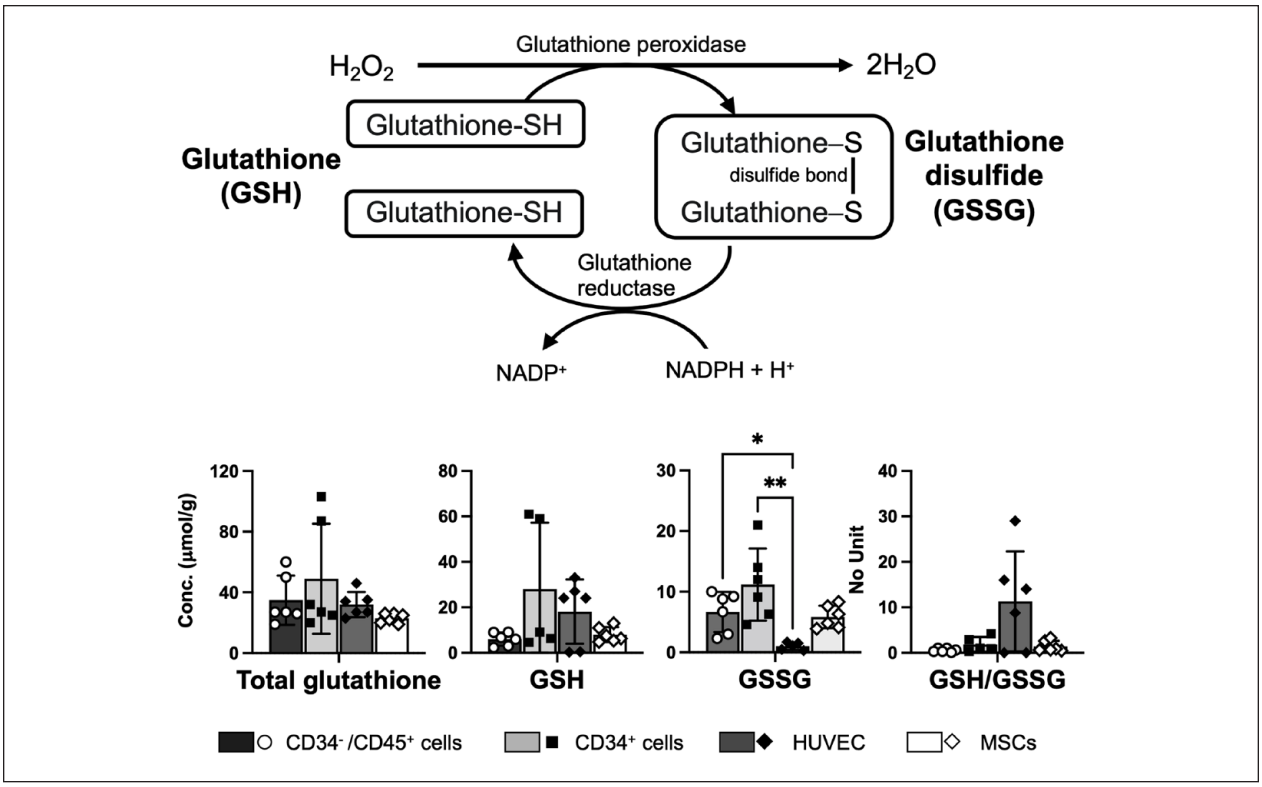

Figure 6. Glutathione redox ratio and total glutathione. The total glutathione level tended to be the highest in $CD34^+$ HSCs. However, the GSH/GSSG ratio was the highest in HUVECs and significantly lower in $CD34^-/CD45^+$ white blood cells and MSCs. Results were expressed as mean ± standard error of mean (SEM) (n = 6). UVECs: human umbilical vein vascular endothelial cells; MSCs: mesenchymal stem cells; GSH: glutathione; GSSG: glutathione disulfide; HSCs: hematopoietic stem cells.
* indicates statistical significance. *$P < 0.05$.

transport of all glycolytic substrates and amino acids from HSCs to vascular endothelial cells along the concentration gradient is likely to activate energy metabolism in the vascular endothelial cells. To date, the selectivity of molecules transported through gap junctions has not been clarified. Therefore, the next objective is to determine which from the large number of small-molecule compounds in HSCs are preferentially transported into vascular endothelial cells and which molecules are the most important and directly relate to the therapeutic effects of HSCs in cell therapies for stroke.

The circulating non-hematopoietic WBCs show lower adenylate energy charge than HSCs, which could be attributed to increased energy consumption or decreased energy production. The $NADPH/NADP^+$ ratio was high in circulating non-hematopoietic WBCs. $NADP^+$ is converted to NADPH mainly by the pentose phosphate pathway, and a high $NADPH/NADP^+$ ratio indicates the activation of the pentose phosphate pathway. In this study, the pentose phosphate pathway was activated to a greater extent in non-hematopoietic WBCs compared with HUVECs or MSCs (Fig. 5). It is unlikely that energy production is decreased in this pathway because the pentose phosphate pathway is involved in the *de novo* synthesis of purine nucleotides. Furthermore, as shown in Fig. 4B, the levels of PRPP and IMP, which are involved in the *de novo* synthesis of purine nucleotides, tended to be higher in CD34-negative WBCs than in HUVECs or MSCs. The *de novo* synthesis of purine nucleotides requires a large amount of ATP for the conversion of PRPP to IMP. Therefore, it is likely that energy consumption is increased in circulating non-hematopoietic WBCs. These results suggest that circulating non-hematopoietic WBCs had a low-energy status because they produced and consumed ATP. Studies have shown that HSCs use the glycolytic system for energy production, whereas WBCs differentiated from HSCs use the glycolytic system for catabolism by switching on oxidative phosphorylation upon differentiation[36]. Pluripotent embryonic stem cells require very high fluxes for glucose uptake and lactate production, even when grown under aerobic conditions[37]. In contrast, the rate of aerobic glycolysis is low in differentiated cells from HSCs and most of the cytoplasmic pyruvate is transferred to the mitochondria, where it is oxidized via the TCA cycle to synthesize ATP. The results of the present study also support these previous findings.

When circulating non-hematopoietic WBCs and HUVECs were co-cultured, the fluorescent small molecules encapsulated

in the WBCs were transferred to HUVECs (Supplemental Figure). This result demonstrates that WBCs interact with vascular endothelial cells and HSCs. However, the results of the *in vitro* assay showed that WBCs could not activate vascular endothelial cells (Supplemental Table). Multiple metabolites were found in higher concentrations in WBCs than in vascular endothelial cells. The results of the *in vitro* assay, however, showed that $CD45^+$ was not able to activate vascular endothelial cells. These results will need to be examined in greater depth. These results suggest that the transfer of a specific low-molecular compound does not lead to the regeneration of blood vessels, although the integrated transfer of various small molecules might lead to the activation of energy metabolism in endothelial cells.

In HUVECs, the lactate/pyruvate ratio was higher, whereas the glycerol 3-phosphate/DHAP ratio and NADH/NAD+ ratios were lower. These results may reflect the intracellular redox potential; indeed, vascular endothelial cells are known to have more oxidative metabolism than other cells, especially stem cells. In addition, the ratio of GSH/GSSG ratio was remarkably high, which might be linked to the high amounts of reduced glutathione. Metabolic state in HUVECs may be a compensatory mechanism of the cell to cope with the oxidative metabolism.

Although the total adenylate content of MSCs was significantly lower than that of HSCs, the intracellular energy charge of MSCs was high and equal to that of the HSCs. In addition, both HSCs and MSCs showed cell–cell interactions with vascular endothelial cells, although their effects on vascular endothelial cells were completely different. HSCs activate the energy metabolism of vascular endothelial cells by providing low-molecular energy sources, like glucose or amino acids, to the energy-depleted endothelial cells, whereas MSCs stabilize their metabolism by absorbing excess energy from vascular endothelial cells overactivated by inflammatory reactions[7,8]. What causes this difference? Hematopoietic stem cells showed significantly higher concentrations of glycolytic substrates or amino acids than normal vascular endothelial cells. This result suggests that ischemia-induced depletion of energy would lead to further concentration differences between HSCs and HUVECs, leading to enhanced transport of small molecules from HSCs to HUVECs through cell–cell interactions. In contrast, the amount of several metabolites and the total energy content were similar in HUVECs and MSCs. In such a case, the concentration difference between HUVECs and MSCs may not be able to drive the transfer of small molecules from MSCs to HUVECs, even if energy depletion occurs due to ischemia.

MSCs are recognized to be equivalent to cerebral vascular pericytes, which act as regulators of vascular endothelial cells[38,39]. The metabolic state of MSCs and HUVECs showed a similar trend, which might be due to the constant communication between MSCs and the HUVECs through the basement membranes of the latter. That is, under normal conditions, the regulation of MSCs (or pericytes in this case) are in constant contact with vascular endothelial cells and regulate each other. When the metabolism of vascular endothelial cells is dysregulated due to inflammatory reactions, the existing pericytes are unable to ameliorate the dysregulation. Hence, it is postulated that the administered exogenous MSCs may help restore normal metabolic function in the vascular endothelial cells by taking up excess energy from the inside of blood vessels. We hypothesize that MSCs are programmed to quell the excessive response of vascular endothelial cells during inflammation by transferring their excess energy to MSCs. Indeed, this may be one of the mechanisms of the anti-inflammatory response of MSCs against graft versus host disease and Crohn's disease.

HSCs and MSCs are the major cell sources in cell therapies, and both stem cell types ameliorate motor dysfunction after stroke. For both HSCs and MSCs, gap junction–mediated cell–cell interaction is one of the major pathways for the treatment of stroke. However, the metabolic states of the two stem cell types tend to be completely different, and the optimal timing of cell therapy is also different because of the different therapeutic mechanisms for cerebrovascular endothelial cells after stroke. The administered HSCs donate a large number of small molecules to the damaged cerebral vascular endothelial cells to activate the brain's energy metabolism and lead to its functional improvement. This means the subacute phase, when the inflammatory response has calmed down, is the optimal time for HSC administration. In contrast, MSCs, such as pericytes, are in constant contact with vascular endothelial cells and regulate each other under normal conditions. However, when the metabolism of vascular endothelial cells is dysregulated due to inflammatory reactions, the existing pericytes are unable to ameliorate the dysregulation. Hence, it is postulated that the administered exogenous MSCs may help restore normal metabolic function in the vascular endothelial cells by taking up excess energy sources from the inside of blood vessels. This means that the optimal time to administer MSCs in the treatment of cerebral infarction is during the acute phase, when the inflammatory response is active. As recognized in the guidelines for the treatment of cerebral infarction[40], it is essential to use the optimal method and time of administration in order to properly assess the therapeutic effect.

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Author Contributions

Conception and design of the study: YO, AK-T, and AT. Cell preparation: AK-T and KY. Analysis and interpretation of data: YO, RA, and OS. Drafting the manuscript: YO, AF, KY, MT, TK, and AT. All authors have approved the final manuscript.

Ethical Approval

Ethical approval for the conduct and report this study was obtained from the research ethics committee of the Foundation for Biomedical Research and Innovation regarding the research use of human umbilical cord blood (approval number: 19-04).

Statement of Human and Animal Rights

All animal experiments were approved by the Animal Care and Use Committee of Foundation for Biomedical Research and Innovation (approval number: 16-03) and complies with the Guide for the Care and Use of Animals published by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

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