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Effect of Human WEE1 and Stem Cell Factor on Human CD34$^+$ Umbilical Cord Blood Cell Damage Induced by Chemotherapeutic Agents

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Abstract Myelosuppression is one of the major side-effects of most anticancer drugs. To achieve myeloprotection, one bicistronic vector encoding anti-apoptotic protein human WEE1 (WEE1Hu) and proliferation-stimulating stem cell factor (SCF) was generated. In this study, we selected human umbilical cord blood CD34$^+$ cells as the *in vitro* model in an attempt to investigate whether *WEE1Hu*, rather than conventional drug-resistant genes, can be introduced to rescue cells from the damage by chemotherapeutic agents such as cisplatin, adriamycin, mitomycin-c and 5-fluorouracil. Cell viability and cytotoxicity assay, colony-forming units in culture assay and externalization of phospholipid phosphatidylserine analysis showed that the expression of *WEE1Hu* and *SCF* in CD34$^+$ cells provided the cells with some protection. These findings suggest that the expression of *WEE1Hu* and *SCF* might rescue CD34$^+$ cells from chemotherapy-induced myelosuppression.

Keywords WEE1Hu; SCF; myelosuppression; radiochemotherapy

Radiochemotherapy (RCT) is an effective treatment for various cancers. But the clinical dosages of chemotherapeutic agents or gamma rays are limited to a subtherapeutic level by myelosuppression, one of the most frequent and severe side-effects of RCT [1]. Introduction of drug-resistant genes into bone marrow cells of cancer patients can protect normal hematopoietic cells from the toxicity of anticancer agents and has been proposed to overcome the dose limitation. The best characterized human multiple drug-resistantce gene (MDR1) confers resistance to multiple structurally dissimilar hydrophobic drugs [2,3]. Mutant forms of dihydrofolate reductase confer resistance to methotrexate and trimetrexate [4,5]. O^6-methylguanine transferase prevents the formation of DNA interstrand cross-links induced by the chloroethylnitrosoureas [6]. Cells overexpressing anti-apoptotic gene *Bcl-2* become resistant to various chemotherapeutic agents [7]. Gene transfer of the rat glutathione S-transferase (GST) *Yc* gene into the hematopoietic system *in vitro* resulted in a 5- to 10-fold increase in resistance to chlorambucil and mechlorethamine [8]. Theoretically, any gene that protects cells against the toxic effects of RCT should be useful for this purpose.

Because of different mechanisms, MDR1 or its family members have no resistance to water-soluble anticancer drugs [9,10], alkylating agents or radiation [11], and the chemoprotective property of the MDR1 gene varies significantly among cell types [12]. Dihydrofolate reductase mutant forms only show resistance to antifolates, and O^6-methylguanine transferase only to nitrosoureas [13]. *Bcl-2* overexpression leads to an uncontrollable increase in the number of cells and has a close relationship with oncogenesis [14]. Transfection of the GST isoform cDNAs into cell lines which have low intrinsic GST-specific activities did not produce convincing resistance to anticancer drugs [13].

Many conventional chemotherapy agents and irradiation frequently cause DNA damage, or block DNA repair, which is one of the major pathways through which chemotherapy causes cell death [15]. Therefore, the human WEE1

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(*WEE1Hu*) is a proper candidate gene for irradiation and chemotherapy-associated suppression of normal hematopoiesis. In response to DNA damage, WEE1Hu can inactivate the Cdc2/CyclinB complexes to activate the G_2 DNA damage checkpoints and then inhibit the G_2/M transition in the cell cycle to prevent cells from undergoing mitosis prior to completion of repair. Incompletion of DNA repair often causes an irreversible alteration to the genome, which was proved to have profound effects on cellular viability, proliferation and development [16]. WEE1Hu might serve as a key regulator of both HIV type 1 Vpr- and gamma irradiation-mediated apoptosis and possibly serve as a general regulator linking the cell cycle to some pathways of apoptosis [17]. The *WEE1Hu* gene was significantly suppressed in tumor cell lines, suggesting its potential role as a tumor suppressor [18].

Hematopoiesis is regulated not only by the proportion of apoptosis and proliferation but also by signals coupling cell growth and differentiation [19]. Conventional gene therapy for myeloprotection provides protection for hematopoietic stem/progenitor cells (HSPCs) from undergoing apoptosis but neglects to maintain and promote their proliferation and growth. Stem cell factor (SCF) is a hematopoietic growth factor showing substantial synergistic activity with a number of other cytokines on primitive HSPCs [20]. Hence, introduction of SCF is able to help hemetopoitic cells repopulate the hematopoietic lineage so as to maintain proper function in bone marrow.

Herein, a bicistronic vector encoding both WEE1Hu and SCF was constructed to investigate whether the introduction of WEE1Hu and SCF into human $CD34^+$ umbilical cord blood (UCB) cells could decrease HSPC death induced by chemotherapy *in vitro* and subsequently augment their proliferation and differentiation. For the first time it was indicated that WEE1Hu and SCF could facilitate the survival and suppress the apoptosis of human $CD34^+$ UCB cells challenged by chemotherapeutic agents *in vitro*.

Materials and Methods

Enrichment and isolation of $CD34^+$ human UCB cells

After informed consent was given by the parent(s), UCB was obtained at the end of full-term deliveries. Mononuclear cells (MNCs) were isolated from UCB using Ficoll-Hypaque (Second Shanghai Chemical Reagent Factory, Shanghai, China) gradient centrifugation. The $CD34^+$ mononuclear cell fraction was isolated with super-paramagnetic microbeads positive selection using high-gradient magnetic field and midi-MACS columns (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. The $CD34^+$ cells were cultured in Iscove's modified Dulbecco's medium (Gibco BRL, Carlsbad, USA) supplemented with 20% heat-inactivated fetal bovine serum (Gibco BRL), 50 ng/ml thrombopoietin (TPO; PeproTech, London, UK) and 50 ng/ml Flt3 ligand (FL; PeproTech) at 37 ºC and 5% CO_2 in a humidified incubator [21].

Plasmid and transfection

The bicistronic plasmid pWEE1Hu-IRES-SCF/EGFP (pWISG) was generated by cloning cDNA enconding WEE1Hu and soluble SCF into pIRES vector (BD Biosciences, San Jose, USA) [22]. The plasmid was prepared using an EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) for transfection.

After prestimulation with human TPO and FL for 48 h, $CD34^+$ cells were resuspended in 400 μl electroporation buffer (Eppendorf, Hamburg, Germany), then 30 μg endotoxin-free plasmid DNA was added. After the pulse (V=400 V, t=150 μs), the cell suspension was wcarefully transferred into Iscove's modified Dulbecco's medium (supplemented with 20% fetal bovine serum, TPO and FL).

Flow cytometry analysis for WEE1 expression

$CD34^+$ cells (4×10^3) were incubated with anti-WEE1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4 ºC. Following washing with phosphate-buffered saline, PE-conjugated goat anti-mouse immunoglobulin G (Jingmei Biotech, Shenzhen, China) was added. Samples were analyzed by a FACSCalibur flow cytometer (BD Biosciences).

Drugs and groups

All the reagents were used at the following recommended peak plasma concentrations: 4 μg/ml cisplatin (Sigma-Aldrich, St. Louis, USA); 8 μg/ml adriamycin (Main Luck Pharmaceuticals, Shenzhen, China); 4 μg/ml mitomycin-c (Kyowa Hakko Kogyo, Tokyo, Japan); and 60 μg/ml 5-fluorouracil (Renmin Pharmaceutical Company, Tianjin, China). They were used alone but not in combination. In every individual experiment, cells were divided into four groups: untreated group, drug only (drug) group, empty vector transfection plus drug (pIRES+drug) group, and recombinant plasmid pWISG transfection plus drug (pWISG+drug) group.

Cell viability assay

$CD34^+$ UCB cells were assayed for their viability using

a CellTiter-Blue Cell Viability kit (Promega, Madison, USA) according to the manufacturer's protocol. The average of fluorescence values was measured and the percent viability was calculated using the following equation:

Percent viability=100%×(experimental–culture medium background)/(maximum fluorescence values–culture medium background)

Cytotoxicity assay

Lactate dehydrogenase release assay was used to measure the percentage of dead cells using a CytoTox-ONE kit (Promega) following the manufacturer's recommendation. After fluorescence was recorded, the percent cytotoxicity was calculated.

Colony-forming unit assay

The clonogenic assay was carried out using a human methylcellulose complete media kit (R&D Systems, Abingdon, UK) as follows. Parental and transfected $CD34^+$ UCB cells were seeded in duplicate in 35 mm Petri dishes (BD Biosciences) at a concentration of 2000 cells/dish in methylcellulose culture medium and cytokine cocktail (50 ng/ml rhSCF, 10 ng/ml rhGM-CSF, 10 ng/ml rhIL-3, 3 U/ml rhEpo) with drugs at concentrations previously determined. Colony scoring was carried out on day 12 for BFU-E (erythropoietic burst formation) and on day 14 for CFU-GM (colony forming unit-granulocyte-macrophage). Colonies expressing at least 50 cells were counted.

Flow cytometry analysis for apoptosis

Apoptotic cells were detected using an Annexin V-fluorescein-isothiocyanate (FITC) and propidium iodide (PI) kit (BD Biosciences) according to the protocol. Annexin V-FITC and PI negative cells are viable, Annexin V-FITC positive and PI negative cells are identified as being in early apoptosis, and cells that are in late apoptosis or already dead are both Annexin V-FITC and PI positive.

Statistical analysis

Data are presented as mean±SD. Wilcoxon matched pairs test was used for the colony-forming study.

Results

Construction and coexpression of bicistronic plasmid pWISG

The construction of pWISG and the coexpression and localization of WEE1Hu and SCF have been reported [22]. The structure of the generated bicistronic expression cassette containing the *WEE1* gene followed by the internal ribosome entry site (*IRES*) sequence and *mhSCF/EGFP* gene is shown in **Fig. 1**.

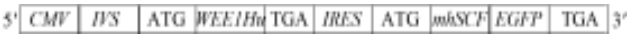

Fig. 1 Bicistronic expression cassette of plasmid pWISG
The elements of the cassette are as follows: CMV, human cytomegalovirus immediate-early promoter/enhancer; IVS, intervening sequence; ATG, initiation codon; WEE1Hu, human anti-apoptotic protein WEE1; TGA, termination codon; IRES, internal ribosome entry site coding sequence; mhSCF, extracellular domain of stem cell factor coding sequence; EGFP, enhanced green fluorescence protein coding sequence.

WEE1 expression

Flow cytometry (FCM) analysis results showed that the percentages of red fluorescence emitting cells in control groups were 0.67% and 0.55%. However, that percentage rose to 40.6% in pWISG transfected $CD34^+$ UCB cells (**Fig. 2**). The application of IRES ensured the co-expression of WEE1Hu and SCF in pWISG [23]. So it was estimated that WEE1Hu and SCF were expressed in more than 40% of $CD34^+$ UCB cells following transfection.

pWISG transfected $CD34^+$ UCB cells obtained increased viability

After 24 h incubation with drugs, the percentages of viable $CD34^+$ UCB cells were determined by measuring their metabolic capacity to reduce resazurin into resorufin [24]. Results shown in **Table 1** indicate that the percent viability of untreated $CD34^+$ cells was 84.17%, and the percentage decreased to a significantly lower rate after exposure to drugs (8.03%–16.06%). pWISG transfected $CD34^+$ cells were approximately 6-fold more resistant to cisplatin than the drug group and mock-transfected control cells (47.13% versus 8.03% and 9.21%, respectively), although it was lower than the vehicle control. pWISG transfected $CD34^+$ cells were only 1.4- to 2-fold more resistant to adriamycin than the drug group and the mock-transfected control (12.74% versus 8.96% and 6.57%, respectively). The fold resistance to mitomycin-c and 5-fluorouracil varied between the former two numbers.

Transfected $CD34^+$ cells lowered their death rate

After confirming that WEE1Hu and SCF expression

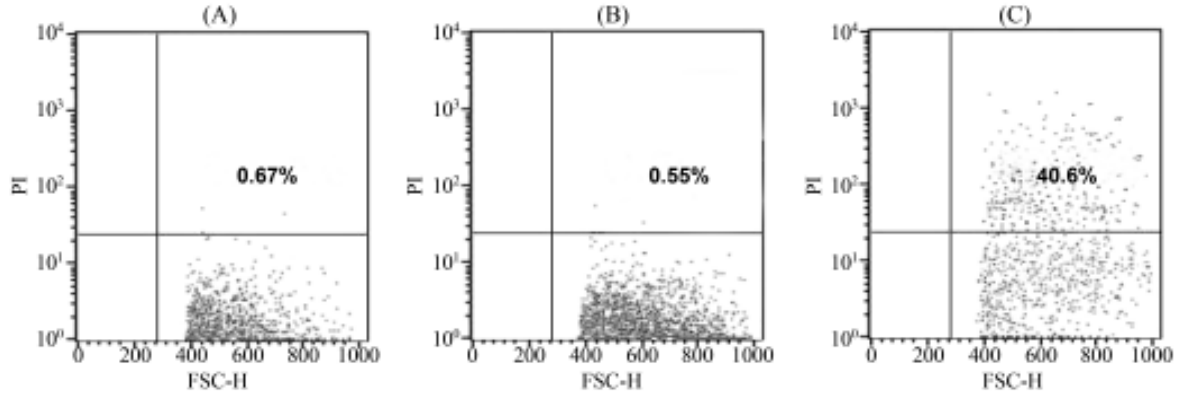

Fig. 2 Flow cytometry (FCM) analysis for WEE1Hu expression

(A) Blank control. (B) pIRES mock control. (C) pWISG tansfected CD34$^+$ UCB cells. Indirect immunofluorescence staining for FCM. Dilution of primary antibody was 1:500. Dilution of PE-labeled secondary antibody was 1:200. FSC-H, forward scatter height.

Table 1 Viability of human CD34$^+$ umbilical cord blood cells incubated with chemotherapy drugs

Group	Untreated (%)	Drug (%)	pIRES+drug (%)	pWISG+drug (%)
Cisplatin	84.17	8.03	9.21	47.13*
Adriamycin	84.17	8.96	6.57	12.74
Mitomycin-c	84.17	16.06	21.62	53.27*
5-Fluorouracil	84.17	10.86	32.11	43.54*

Forty-eight hours after electroporation, drugs were added. Thirty hours later, the viability of CD34$^+$ cells was determined by a CellTiter-Blue Cell Viability kit. The data are presented as mean percentages from triplicate wells. * P<0.05 versus pIRES+drug groups and only drug groups. The experiment was repeated twice, and similar results were obtained.

could promote the viability of CD34$^+$ cells, we also examined whether these two genes correlated with inhibition of cell death. Membrane integrity was analyzed by measuring the release of lactate dehydrogenase from damaged CD34$^+$cells. After 48 h incubation with drugs, the death rate of CD34$^+$ cells rose sharply, from 9.87%–10.28% to approximately 60%. At a concentration that was presumed to be the maximum adriamycin concentration in the bone marrow, the death rate of pWISG transfected CD34$^+$ cells was nearly one-third that of the drug control and mock-transfected control (24.00% versus 65.12% and 79.50%, respectively). When exposed to cisplatin, mitomycin-c and 5-fluorouracil, pWISG transfected CD34$^+$ cells also had a reduced death rate compared with drug groups and mock-transfected control groups (**Table 2**).

Table 2 Cytotoxicity mediated by different chemotherapy drugs against human CD34$^+$ umbilical cord blood (UCB) cells

Group	Untreated (%)	Drug (%)	pIRES+drug (%)	pWISG+drug (%)
Cisplatin	9.87	60.58	42.53	30.97*
Adriamycin	9.87	65.12	79.50	24.00*
Mitomycin-c	10.28	63.67	56.74	40.91*
5-Fluorouracil	10.28	52.22	62.88	33.74*

Forty-eight hours after electroporation, drugs were added. Thirty-six hours later, the cytotoxicity of CD34$^+$ UCB cells was determined by lactate dehydrogenase release assay. Results represent the mean percentage cytotoxicity from triplicate wells. CD34$^+$ UCB cells were enriched from four independent separations. * P<0.05 versus pIRES+drug groups and only drug groups. The experiment was repeated twice, and similar results were obtained.

Multilineage potential of gene modified CD34$^+$ UCB cells

Whether CD34$^+$ UCB cells survive in response to drugs was committed to either the myeloid (GM) or erythroid (E) cell lineages or whether they were multipotent. After 12–14 d incubation in methylcellulose complete media, cultures were scored for GM and E colonies. Through this assay we were able to show that the numbers of CFU-GM and BFU-E treated by pWISG transfection plus drugs were more than that of the drug groups and mock-transfected groups. Significant differences of BFU-E numbers between these groups occurred when cells were exposed to cisplatin (P<0.05, 62.33±5.03 versus 48.33±1.53 and 45.00±2.65, respectively) [**Fig. 3(A)**], mitomycin-c (P<0.05, 76.67±2.51 versus 51.33±7.02 and 37.67±5.13, respectively) [**Fig. 3(C)**] or 5-fluorouracil (P<0.05, 55.33±2.08 versus 39.00±2.65 and 25.33±4.73, respectively) [**Fig. 3(D)**], but not to adriamycin. However, significant differences of CFU-GM numbers occurred only when cells were exposed to adriamycin (P<0.05, 16.33±3.51 versus 5.66±1.15 and 4.88±0.58, respectively) [**Fig. 3(B)**] and to 5-fluorouracil (P<0.05, 17.00±3.00 versus 8.33±0.58 and 7.00±4.58, respectively) [**Fig. 3(D)**].

WEEHu and SCF suppress apoptosis of CD34$^+$ UCB cells

In our study, WEEHu and SCF expression has been shown to counteract the toxic effect of chemotherapy drugs on CD34$^+$ UCB cells by supporting cell viability, inhibiting cell death and maintaining pleiotropic properties. To determine whether the increased viability and decreased death rate of CD34$^+$ cells correlated with suppression of apoptosis, cells were examined for apoptosis. As apoptotic hematopoietic cells rapidly undergo secondary necrosis and disintegrate in culture [25], we chose the 24 h time point, rather than the time point for most pronounced decrease in viability (44 h), to study early apoptosis. Apoptosis was assayed and compared among the different groups. As shown in **Fig. 4**, there was a significant anti-apoptotic effect on pWISG transfected CD34$^+$ cell groups compared to drug groups and mock-transfected groups when challenged with each of the four drugs. The apoptotic percentage of CD34$^+$ induced by cisplatin was 19.10%, adriamycin 7.29%, mitomycin-c 19.28%, and 5-

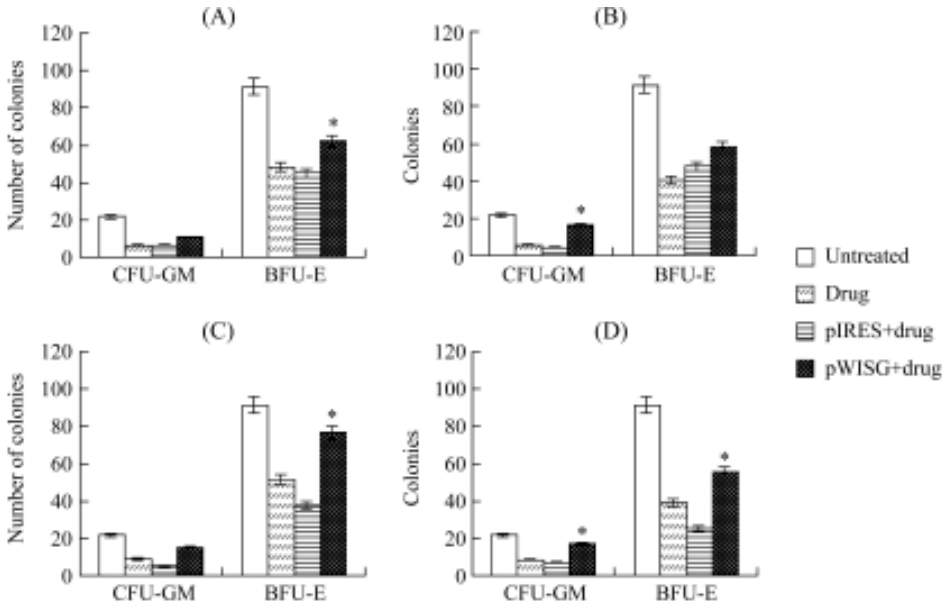

Fig. 3 Multilineage potential of human CD34$^+$ umbilical cord blood cells surviving in response to cisplatin (A), adriamycin (B), mitomycin-c (C) and 5-fluorouracil (D)

CD34$^+$ cells were seeded at a density of 2000 cells per 35 mm Petri dish and incubated in methylcellulose culture supplement with a cytokine cocktail and anticancer drugs at a maximun concentration in blood plasma. All samples were set in duplicate. After 12–14 d of incubation at 37 ºC and 5% CO_2 in air, clones containing 10 or more cells were counted by microscopy. The numbers of drug-resistant colonies are presented as the mean±SD of three individual experiments. * Significant increment compared with the drug group and mock-transfected control group as determined by Wilcoxon test (P<0.05). BFU-E, erythropoietic burst formation; CFU-GM, colony forming unit-granulocyte-macrophage.

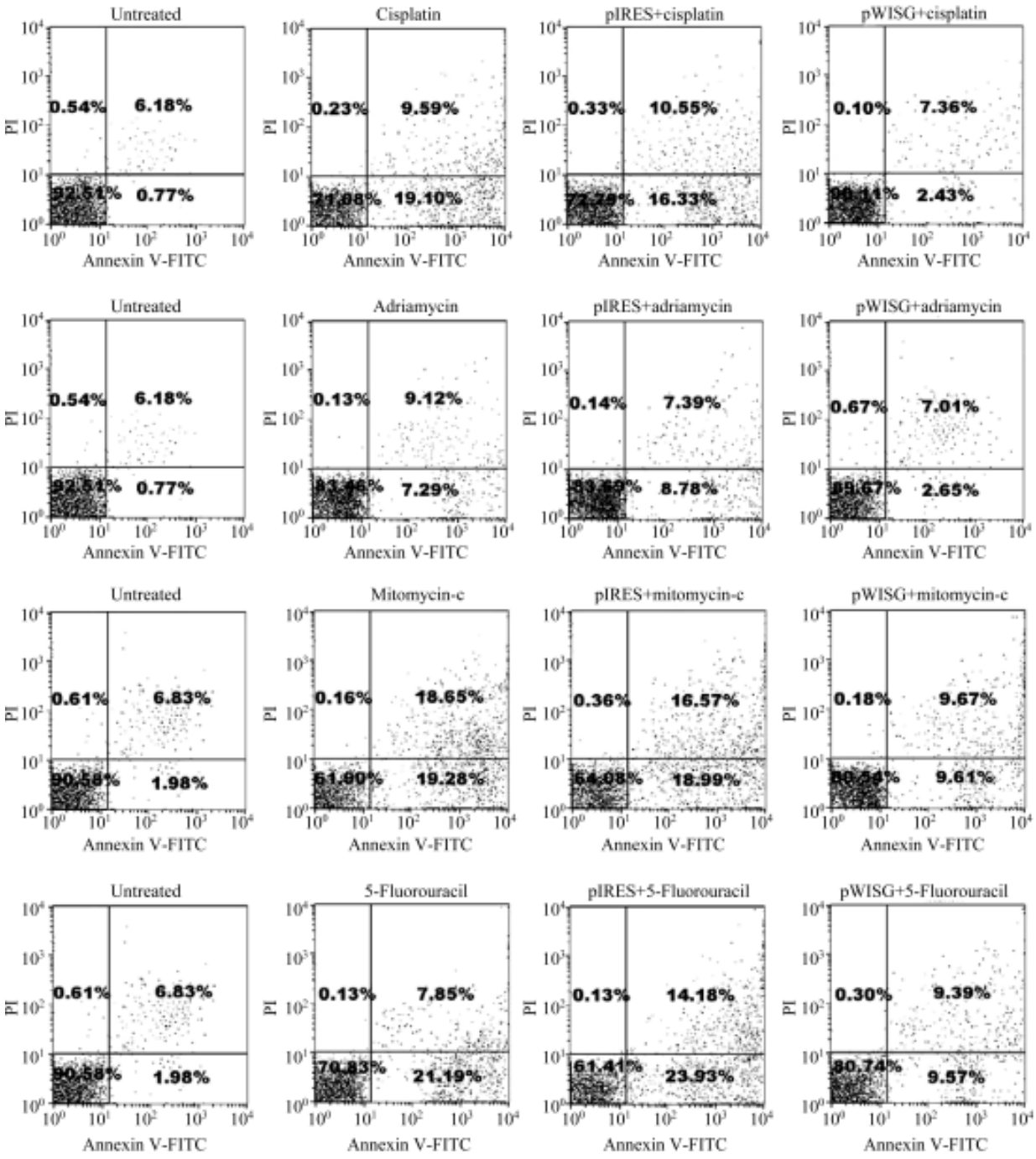

Fig. 4 Human WEE1 and stem cell factor counteract apoptosis of human CD34$^+$ umbilical cord blood cells

After pretreatment, 5×10^3 CD34$^+$ cells were incubated in medium with or without drugs for 24 h. Then cells were rinsed twice with phosphate-buffered saline and stained with Annexin V-fluorescein-isothiocyanate (FITC) and propidium iodide (PI). Figures in the lower right quadrant of each graph indicate the percentage of apoptotic cells which are Annexin V positive and PI negative. CD34$^+$ cells were enriched from three independent separations. Results are from one of two experiments, with similar results.

fluorouracil 21.19%. These figures dropped to 2.43%, 2.65%, 9.61% and 9.57%, respectively, when combined with pWISG transfection. There was no significant protection from pWISG transfection on late apoptosis induced by cisplatin, adriamycin or 5-fluorouracil. Our data strongly revealed that WEEHu and SCF overexpression

suppressed $CD34^+$ cell apoptosis induced by these four chemotherapeutic drugs.

Discussion

Cisplatin, adriamycin, mitomycin-c and 5-fluorouracil have broad spectra of antitumor activity and are included in standard front-line treatment regimens for a variety of adult and pediatric tumors. However, the chemotherapy drug associated myelosuppressive effect restricts the applicable dosages and often demands delay or intermittence during the treatment for hematopoietic recovery. DNA is the primary cellular target for the damaging effects of most chemotherapeutic agents [15, 26]. Thus, after high-dose chemotherapy, transferring genes that could protect DNA from damage, such as *WEE1Hu*, into hematopoietic cells with subsequent transplantation might provide myeloprotection against these agents and allow post-transplant chemotherapy to be safely used. This could be an important strategy that might prevent the need for dose delay or allow for the use of post-transplant chemotherapy, possibly leading to improved response rates after high-dose chemotherapy and hematopoietic cell transplantation.

Depending on the planned treatment duration, the targeted cells, their location, and whether they undergo cell divisions or no longer divide, one can use non-viral methods, non-integrating viral vectors, or integrating viral vectors for gene transfer. However, in recent years the clinical application of these methods has advanced more slowly than expected for different reasons. Non-viral methods of delivery are less efficient than viral methods. For hematopoietic stem cells and other stem cells that must divide to regenerate blood or other organs, an integrating vector is required. But risks of insertional mutagenesis with integrating viral vectors remain the biggest concern in gene transfer *in vivo*. Non-integrating viral vectors do not integrate into chromosomes and could enter into non-dividing hematopoietic stem cells, but elicit immune responses in humans [27,28].

Gene therapy as applicable to myelosuppression will probably require only transient expression of the desired protein, perhaps into committed progenitor cells. Thus, the $CD34^+$ cells seem to be the ideal targets for myeloprotection [13]. For this purpose, a bicistronic plasmid encoding human WEE1 and SCF using IRES was constructed and transfected into human $CD34^+$ UCB cells. In our previous study, it was shown by immunohistochemistry and Western blot analysis that WEE1Hu protein was expressed and located in the nucleus. The expression and receptor-binding ability of the SCF were determined by reverse transcription–polymerase chain reaction and flow cytometry. The previous study also suggested that the introduction of two functional genes using a bicistronic vector was more powerful and efficient than single gene therapy [22].

In this study, we first examined the viability of $CD34^+$ cells and found that nearly half of pWISG transfected $CD34^+$ progenitor cells survived in response to cisplatin, mitomycin-c and 5-fluorouracil, whereas a very small portion of negative control cells survived. However, the effect was not obvious in response to adriamycin. This experiment showed that, when exposed to different anticancer drugs, *WEE1Hu* had a selective viability-promoting effect on $CD34^+$ progenitor cells. In the following colony-forming cell assay, the pWISG transfected $CD34^+$ cells surviving in response to 5-fluorouracil retained multilineage potential to be committed to myeloid and erythroid cell lineages in comparison with controls. Only erythroid colony numbers significantly increased in response to cisplatin and mitomycin-c compared with two negative controls. When exposed to adriamycin, only myeloid colony numbers significantly increased. Because the human methylcellulose complete media was supplemented with sufficient rhSCF, in this assay, the increased CFU-GM and BFU-E colony formation of pWISG transfected $CD34^+$ cells in the presence of drugs suggested the role of WEE1Hu in promoting cell viability, but not the function of SCF. It was unclear whether *WEE1Hu* selectively protected erythroid or myeloid hematopoietic progenitor cells from damage after exposure to different anticancer drugs. The disparity in colony-forming capacity of pWISG transfected $CD34^+$ cells in response to different chemotherapeutic agents might be due to the transient and unstable expression of transferred *WEE1Hu* in $CD34^+$ cells. The colony forming cell assay lasted for 14 d, whereas transient expressed *WEE1Hu* could last for no more than 12 d (estimated by duration of co-expressed SCF) [29] in $CD34^+$ cells. The *WEE1Hu* protective effect could not cover all the culture periods. Therefore, the colony numbers made no statistical difference compared with controls when induced by some drugs, although the numbers were higher than controls. An adenoviral delivery system should be carried out to undertake further investigation.

The ability of *WEE1Hu* to promote the viability and inhibit the cytotoxicity of $CD34^+$ progenitor cells is likely to be related with the suppression of apoptosis occurring in the decreased sensitivity to DNA damage [30,31]. This

hypothesis was supported by the observation that *WEE1Hu* suppressed apoptosis of $CD34^+$ UCB cells. Because $CD34^+$ UCB cells, like any other enriched progenitor cell population, are somewhat heterogeneous, it remains possible that the apoptotic cells or the viable cells do not necessarily represent progenitor cells. However, our colony forming cell studies investigating the viability of clonogenic progenitor cells support the idea that *WEE1Hu* suppresses apoptosis of $CD34^+$ progenitor cells.

In this study, *WEE1Hu* only conferred several-fold resistance to the four drugs, whereas other drug-resistant genes have been reported to confer 95-fold resistance to chemicals [13]. The gene transfer efficiency could not be corrected as no pronounced drug-resistant gene has been used as a control. Also, there are indications that transfection with pWISG has some toxicity sparing activity.

In conclusion, this study showed that cisplatin, adriamycin, mitomycin-c and 5-fluorouracil induced damage to $CD34^+$ cells. But the expression of *WEE1Hu* and *SCF* in $CD34^+$ cells increased their resistance to the damage induced by these agents. These findings suggest the possibility that expression of *WEE1Hu* and *SCF* in $CD34^+$ cells might enable rescue from myelosuppression to patients receiving these drugs.

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