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RESEARCH ARTICLE

Role of Chk1 in the differentiation program of hematopoietic stem cells

Laura Carrassa · Elisa Montelatici · Lorenza Lazzari · Stefano Zangrossi · Matteo Simone · Massimo Broggini · Giovanna Damia

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Abstract Hematopoietic stem cells (HSC) isolated from umbilical cord blood (UCB) were treated with ionizing radiation (IR) and sensitivity and IR induced checkpoints activation were investigated. No difference in the sensitivity and in the activation of DNA damage pathways was observed between CD133+ HSC and cells derived from them after ex vivo expansion. Chk1 protein was very low in freshly isolated CD133+ cells, and undetectable in ex vivo expanded UCB CD133+ cells. Chk1 was expressed only on day 3 of the ex vivo expansion. This pattern of Chk1 expression was corroborated in CD133+ cells isolated from peripheral blood apheresis collected from an healthy donor. Treatment with a specific Chk1 inhibitor resulted in a strong reduction in the percentage of myeloid precursors (CD33+) and an increase in the percentage of lymphoid precursors (CD38+) compared to untreated cells, suggesting a possible role for Chk1 in the differentiation program of UCB CD133+ HSC.

Keywords Stem and progenitor cells · Umbilical cord blood · Chk1 · DNA damage · Differentiation

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L. Carrassa (✉) · M. Simone · M. Broggini · G. Damia
Laboratory of Molecular Pharmacology,
Department of Oncology, Istituto di Ricerche Farmacologiche "Mario Negri", via La Masa 19, 20156 Milan, Italy
e-mail: laura.carrassa@marionegri.it

E. Montelatici · L. Lazzari · S. Zangrossi
Cell Factory, Centre for Transfusion Medicine,
Cell Therapy and Cryobiology, Department of Regenerative Medicine, Ospedale Maggiore Policlinico,
Mangiagalli e Regina Elena, Milan, Italy

Introduction

The signal transduction pathway activated after DNA damage consists of many molecular components. Two important DNA damage sensor kinases can be distinguished: ataxia telangiectasia mutated (ATM) and ATM- and Rad3related (ATR). These kinases sense the damage and respond by autophosphorylation and phosphorylation of other DNA damage sensors such as H2AX. They also activate other downstream components of the signaling pathway: the transducers Chk1 and Chk2 and the effectors (i.e. p53, Cdc25s) that arrest the cell cycle (in G_1–S, S or G_2–M phases), giving the cell time to repair the damage or, if the damage is irreparable, to undergo apoptosis [1–3]. A functional signal transduction pathway activated in response to DNA damage has the aim of maintaining genomic stability. In this scenario Chk1 is implicated in several checkpoints of the cell cycle providing a crucial link between the upstream sensors of the checkpoints and the cell cycle engine. Chk1 role during normal cell cycle progression remains controversial, although several recent evidences shed light on the essential role of Chk1 both in normal and cancer proliferating cells [4–6]. A role for Chk1 in transcriptional repression after DNA damage has also been described through its involvement in the phosphorylation status of histone H3 [7].

Although DNA signal transduction and repair pathways substantially influence the cytotoxic effects of different anticancer agents in normal and tumor cells, little information is available on these mechanisms in hematopoietic stem/precursor cells (HSPC).

Umbilical cord blood (UCB) is a rich source of HSPC and this makes it particularly suitable for hematopoietic stem cell (HSC) transplantation. Since the first transplant

of HSC from UCB in 1989, there has been increasing interest in using UCB as an alternative source of cells for transplantation, especially in pediatric onco-hematological diseases [8]. The limitation of using UCB in adults, with their larger body weight, is due to the limited number of HSPC present in UCB units [9, 10], making ex vivo expansion of UCB HSPC necessary. In the last few years, several groups have developed ex vivo expansion protocols allowing the transplantation of progenitor cells from cord blood in adults too [11–13]. HSPC have the potential both to self renew for their maintenance over time and to differentiate to all the different specialized cell types of the hematopoietic system [14]. In recent years monoclonal antibodies against CD34 antigen have been useful to identify and isolate HSPC; recently a new cell surface antigen CD133, expressed on a subpopulation of CD34+ HSPC, has shown capacity to reconstitute the entire hematological and immune system of lethally irradiated mice and was also able in vitro to differentiate into neuronal and endothelial cells, indicating a much greater plasticity of CD133+ cells than CD34+ cells [15, 16]. In addition, the immuno-magnetic cell selection procedures and the ex vivo expansion systems have been instrumental for molecularly and biologically characterizing the different compartments of hematopoietic cells, i.e. stem cells and progenitor cells, but studies on their DNA repair capacity and/or DNA damage checkpoint activation are still scant.

Gene expression analysis in human and mouse hematopoietic and neural stem cells has shown that genes involved in DNA repair pathways (mismatch repair, nucleotide excision repair or double strand break repair) were expressed more in stem cells than in more differentiated cell types [17]. Whether and how this relates to a difference in sensitivity of the HSPC population to DNA damaging agents has still to be clarified. Even less is known about the DNA damage response pathways activated in hematopoietic stem cells from UCB and bone marrow (BM) and in a more differentiated progeny derived from them. Some information is available on mouse embryonic stem cells that do not undergo cell cycle arrest after DNA damage but readily undergo p53-independent apoptosis [18]. p53 was barely phosphorylated after ionizing radiation (IR), and this correlated with very low expression of the p53 target gene p21. However, in spite of the lack of cell cycle checkpoints and p53 activity, mouse embryonic stem cells activate ATM and induce the formation of the γH2AX foci which are necessary for activation of the DNA damage response [19].

To improve our understanding of the DNA damage response in hematopoietic stem cells, we isolated CD133+ cells from UCB and compared the IR-induced DNA damage response and checkpoint activation in these cells and in cells derived from them after ex vivo expansion with a specific cytokine cocktail. No difference in the activation of DNA damage checkpoint pathways was observed between CD133+ hematopoietic stem cells and the precursors cells derived from them. Interestingly, in this experimental system Chk1 is not essential for growth and for the cytotoxic response to IR. On the contrary, a possible role of Chk1 in differentiation of HSC is postulated.

Materials and methods

Collection of UCB, apheresis, CD133 purification and cell culture

Umbilical cord blood was collected, after obtaining informed consent, from healthy mothers of full-term newborns ($n = 36$). After delivery, the umbilical cord was clamped and disinfected and UCB was recovered with the placenta in utero into sterile UCB collection bags (MacoPharma) containing 29 mL of citrate–phosphate dextrose (CPD) as anticoagulant. Mononuclear cells (MNCs) were isolated by density gradient centrifugation (Lympholyte-H, 1.077 g/mL; Cedarlane, Hornby, Ontario, Canada) at 400*g* for 30 min at room temperature. The low-density cell fraction was collected and washed twice in Ca^{2+}, Mg^{2+} free Dulbecco's phosphate-buffered saline (PBS; GIBCO) containing 1% bovine serum albumin (BSA; Sigma) and 2 mM of ethylene-diamine-tetra-acetic acid (EDTA, Sigma). The CD133+ cells were isolated from UCB MNCs using a positive immunomagnetic selection system (Miltenyi Biotech, Bergisch Gladbach, Germany), and their purity was analyzed by flow cytometry (FC500, Beckman Coulter, Fullerton, CA, USA). The median purity of CD133+ cells was 92% (87–97). The negative fraction was acquired from the same positive immunomagnetic selection during the isolation of CD133+ from UCB MNCs. The cell separation was optimized finding the optimal concentrations of antibodies and magnetic colloids in order to obtain the highest purity of CD133+ cells, the best recovery and the negative counterpart nearly 100% pure with no stem/progenitor cell contamination. The CD133− negative fraction is then called "differentiated cells". Aliquots of leukapheresis were obtained from healthy peripheral blood stem cell donors, with their informed consent, after mobilization consisting in a 3 days course of granulocyte colony-stimulating factor (G-CSF, 10 μg/kg/day). The HL-60 human promyelocytic acute leukemia cell line was cultured in RPMI 1640 medium (Lonza, Verviers, Belgium), supplemented with 10% of fetal bovine serum.

Ex vivo expansion, IR and drug treatments

After purification, CD133+ cells were seeded at a density of 5×10^3 cells/mL in 24-well plates in a serum free medium (CellGro SCGM medium, Cell Genix) with a cytokine cocktail including thrombopoietin (TPO) (10 ng/mL), Flt3 ligand (FL) (50 ng/mL), Interleukin-6 (IL-6) (10 ng/mL) and stem cell factor (SCF) (50 ng/mL) (Peprotech EC, London, UK) and 10% of AB Human Serum. This cytokine combination allows expansion of both myeloid and megakaryocytic compartments in two weeks with a median (and range) fold expansion of total nucleated cells of 260 (180–310) (Montemurro T. et al., submitted). The cells were incubated for 2 weeks at 37°C in a fully humidified atmosphere with 5% CO_2 and cytokines were replaced every 3 days.

For the IR treatment, cells (at 0, 3 and 14 days from isolation) and terminally differentiated cells were seeded at 2.5×10^4 cells/mL in 24-well plates and treated with different IR doses; 72 h later, treated and untreated cells were counted using a Coulter counter (Beckman Coulter). CHIR-124 compound [20] was used as Chk1 inhibitor and it was synthesized by Dr Patrick Casala (Institut de Recherches Servier, Croissy sur Seine, France). Chk1 inhibitor treatment was done on CD133+ cells seeded at approximately 2.5×10^4 cells/mL on the day of purification from UCB at a concentration representing the IC_{50} for this experimental system (30 nM). Another Chk1 inhibitor, AZD 7762 (Axon Medchem) at a concentration of 3.75 nM was also used [21]. 5-aza-2′-deoxycytidine (Sigma) was given to ex vivo expanded cells at a final concentration of 0.05 μM starting from day 6 from purification. Fresh drug was added every day up to day 14.

FACS analysis

Before and after expansion, CD133+ cells were washed and stained for 20 min at room temperature in the dark with the following monoclonal antibodies: CD34 PE (Becton–Dickinson, BD, Franklin Lakes, NJ, USA.), CD45 PC7 (Beckman Coulter, Fullerton, CA 92834-3100 USA) and CD133 APC (Miltenyi Biotech). Cells incubated with isotypic control-PE, PC7, APC were used as negative controls. The cells were characterized for the expression of the following surface markers: CD19 FITC (BD), CD3 APC (Beckman Coulter), CD61 FITC (BD), CD56 PE (BD), CD33 APC (Beckman Coulter), CD38 FITC (BD). After staining the cells were washed once with PBS containing 0.1% BSA. At least 100,000 events were acquired with a Cytomics FC500 (Beckman Coulter) and analyzed using the CXP-analysis software. A similar staining procedure was performed for the expression of the marker CD 33 in HL 60 cells.

Western blot analysis

Briefly, protein extracts were obtained using a lysis buffer containing 10 mM Tris–HCI pH 7.4, 150 mM NaCI, 0.1% Nonidet NP-40, 5 mM EDTA, 50 mM NaF in the presence of protease inhibitors. As much as 30 μg of total cellular proteins were separated on 8% SDS–polyacrylamide gels and electro-transferred to nitrocellulose membrane (PROTRAN, Schleicher e Schull). Immunoblotting was carried out with anti-Chk1 (G-4), ß-actin (C-11), p53 (DO-1), Chk2 (H-300), P-Cdc25C-ser216, Cdc25C (C-20), p21 (C-19) purchased from Santa Cruz Biotechnology; anti Ran (clone 20) was purchased from BD Transduction Laboratories. Anti-S317 Chk1, anti-S345 Chk1 and anti-T68 Chk2 were purchased from Cell Signaling.

Immunofluorescence analysis

Cells (30–40,000) were fixed in 4% paraformaldehyde and spotted on poly-lysine cover glasses (VWR international, Milan, Italy) overnight. After permeabilization with Triton X-100 (0.5% in PBS) for 5 min cells were blocked in 2% BSA, 0.2% Triton X-100 in PBS (blocking buffer) for one hour then incubated for one hour with the anti Chk1 primary antibody (1:500, clone DCS310, SIGMA) then a Cy3 conjugated secondary antibody (1:500, Alexa Fluor, 594. Molecular Probes) was added. DAPI (Sigma, final concentration 30 ng/mL in PBS) was added for one minute to stain nuclei, and cells were mounted with the mounting media Fluor Save (Calbiochem, Darmstadt, Germany) and observed in a Zeiss Axiophot photomicroscope equipped for epifluorescence (Carl Zeiss, Oberkochen, Germany).

RNA isolation, cDNA preparation and real-time PCR

Total RNA was purified using the SV-Total RNA isolation system (Promega, Milan, Italy). As much as 300 ng of total RNA were reverse-transcribed to cDNA in 20 μL of reaction mix with an Archive Kit (Applied Biosystems, Foster City, USA) using random primers. Real-time PCR was run to quantify Chk1 and actin. Primers and TaqMan probe were purchased as ready-to-use solutions (Assay on Demand, Applied Biosystems). Real-time PCR was done using the 7900HT Sequence Detection System (Applied Biosystems).

Results

Cytotoxicity and activation of DNA damage pathways after IR treatment in CD133+ stem cells isolated from UCB and in cells at different differentiation status

Cytotoxicity after IR was investigated in CD133+ cells purified from UCB and in the ex vivo expanded progeny of cells derived from them after culturing in a specific cytokine cocktail for 14 days. The cytotoxic effect of IR was also evaluated in terminally differentiated cells derived from the corresponding UCB. Cells were irradiated with different doses and growth inhibition was evaluated 72 h later. As shown in Fig. 1a, sensitivity to IR was comparable for the CD133+ stem cells and the ex vivo expanded cells, with an IC_{50} of approximately 2 Gy; the IR IC_{50} was much higher in the completely differentiated UCB cell component (>10 Gy).

After 3 and 14 days of UCB CD133+ cell expansion, the expanded cells were irradiated with different doses and activation of the DNA damage pathways were studied at 1 and 24 h. Figure 1b shows p53 stabilization starting at 1 h and persisting up to 24 h after 5 Gy in cells on day 3 of culture; Chk2 protein kinase was activated, as indicated by the detection of threonine 68 phosphorylated at 1 h and 24 h after irradiation. Similarly also Chk1 protein kinase appears activated as indicated by phosphorylation in serine 345 at both the time points investigated. No p21 induction was observed, even after 5 Gy.

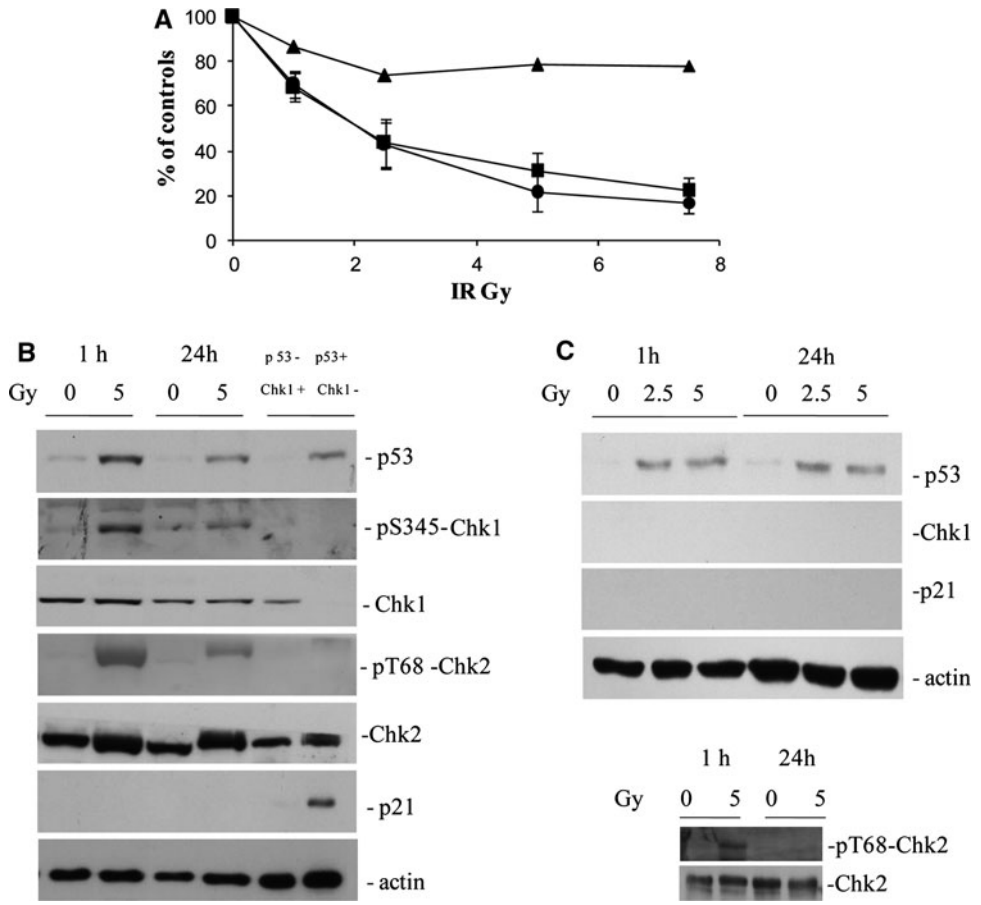

Fig. 1 Cytotoxicity and activation of DNA damage pathways after IR treatment in CD133+ stem cells isolated from UCB and in cells at different differentiation status. **a** Cytotoxic effect of IR in UCB CD133+ cells (*filled circle*), in the ex vivo expanded CD133+ cell population (*filled square*) and in terminally differentiated cells representing the negative fraction after CD133+ isolation (*filled triangle*). Data are expressed as percentages of the untreated control sample; mean ± SD of at least three different UCB donors. **b** Western blot analysis in the cell progeny from UCB CD133+ cells after 3 days of ex vivo expansion with the cytokine cocktail. p53, Chk1, pS345Chk1, Chk2 pT68Chk2, p21 and actin protein levels in control cells and in cells treated with 5 Gy IR and harvested 1 h and 24 h later. *Lane 5* U2OS cells transfected with scramble siRNA (Chk1 positive, p53 negative); *lane 6* U2OS cells transfected with a specific Chk1 siRNA (Chk1 negative, p53 positive). For further details see [5]. **c** *upper panel* Western blot analysis in the cell progeny from UCB CD133+ cells after 14 days of ex vivo expansion with the cytokine cocktail. p53, Chk1, p21 and actin protein levels in control cells and cells treated with 2.5 and 5 Gy IR and harvested 1 h and 24 h from irradiation. *Lower panel* Chk2 and pT68-Chk2 protein levels in control cells and in cells treated with 5 Gy IR

When the same analysis was done on cells ex vivo expanded for 14 days, there was clear activation of p53 and Chk2 (Fig. 1c, right panel), but again no p21 induction. Surprisingly, no Chk1 protein was detectable.

Kinetics of Chk1 expression in CD133+ cells from UCB and ex vivo expanded

The lack of Chk1 protein in the expanded UCB CD133+ cells prompted us to investigate its expression in the initial CD133+ population purified from UCB and in cells at different times during the ex vivo expansion. Cells were collected every other 3 days up to 14 days and proteins were extracted. Western blot analysis indicated that Chk1 protein was almost undetectable on the day of purification, significantly increased and was clearly detectable on day 3, barely detectable on day 6 and completely undetectable at subsequent time points (9, 12 and 14 days) (Fig. 2a). Figure 2b shows densitometric analysis of three independent experiments, as percentages of a positive control sample. These data were corroborated by immuno-fluorescence analysis (Fig. 2c). Chk1 protein was also undetectable in the terminally differentiated hematopoietic cell population (representing the negative fraction after the CD133+ isolation) derived from UCB (Fig. 2d).

To see whether in this experimental system Chk1 down-regulation occurred at transcriptional level, Chk1 mRNA levels were studied by real time PCR at the same time points. Chk1 mRNA levels followed same kinetics as protein expression, suggesting that Chk1 is down-regulated at transcriptional level in these cells. Additionally, to test whether a specific methylation program was active in the Chk1 promoter, cells were either treated or not with 5-aza-2′-deoxycytidine (0.05 μM), which at the doses used has a de-methylating effect, and then mRNA and total proteins were collected at established time points from the beginning of expansion. Figure 3a and b shows there were no appreciable differences between untreated and treated samples.

To investigate whether the Chk1 protein kinetics observed in the UCB CD133+ cells were also seen in another experimental system, CD133+ cells were isolated from peripheral blood (PB) apheresis collected in healthy donor, after 3 days of G-CSF treatment. The cells were cultured with the cytokine cocktail as for UCB CD133+ cells. Figure 4a shows that the pattern of Chk1 expression

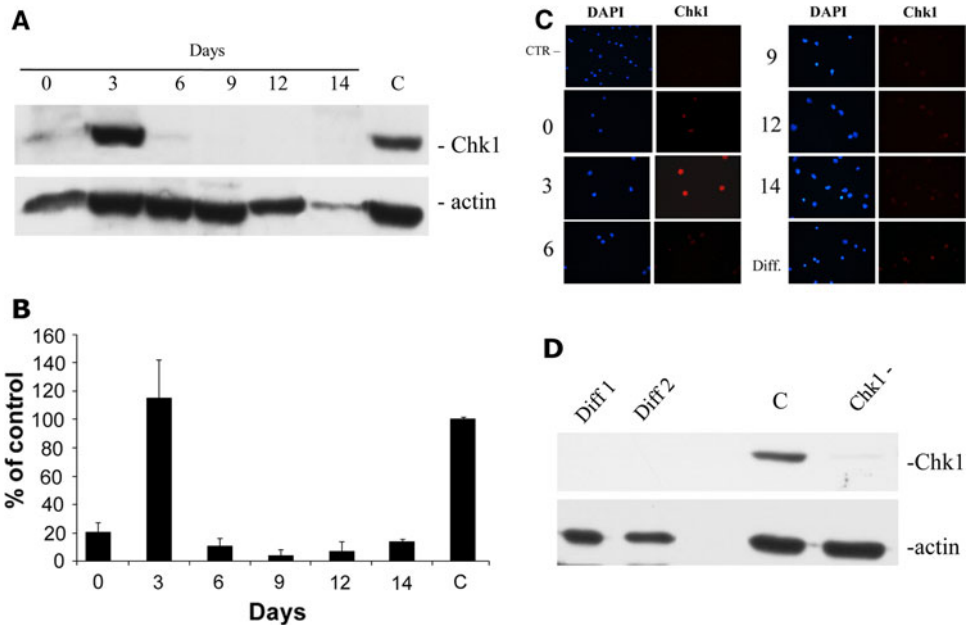

Fig. 2 Kinetics of Chk1 expression in UCB CD133+ cells during 14 days of ex vivo expansion and in negative fraction after the CD133+ isolation. **a** Western blot analysis showing the Chk1 and actin protein levels in samples collected every 3 days starting from the freshly isolated UCB CD133+ cells (day 0) up to day 14. A sample with protein extracts from Jurkat cell lines is also included (*lane C*). **b** Densitometric analysis of the levels of Chk1. Data are percentages of control sample and normalized by the actin protein levels; mean ± SD of three independent experiments. **c** Immunofluorescence analysis of Chk1 in freshly isolated UCB CD133+ cells (day 0) and in the ex vivo expanded cell population. The analysis was also done in terminally differentiated cells, representing the negative fraction after CD133+ isolation (diff.). Ctr—sample comprises cells stained with no primary anti Chk1 antibody. DAPI staining is also shown. **d** Western blot analysis showing the complete absence of the Chk1 protein in terminally differentiated cells. Control sample (C) represents cell extracts from HCT-116 cell line while the Chk1— sample represents cell extracts from HCT-116 cells transfected with siRNA against Chk1

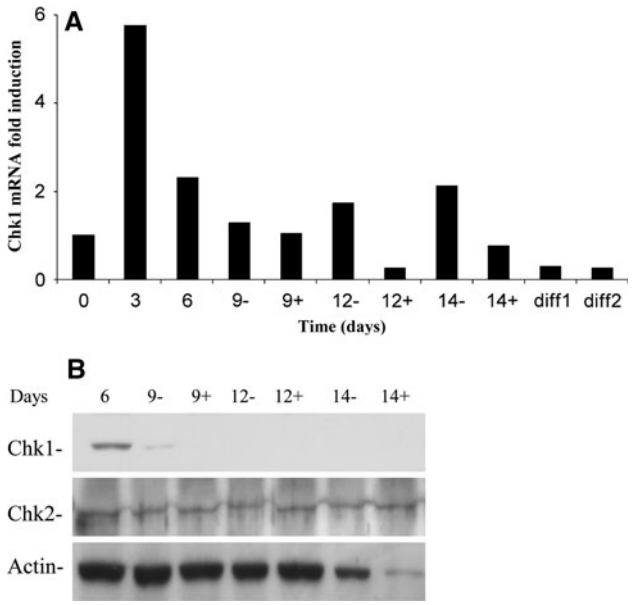

Fig. 3 Chk1 mRNA levels in UCB CD133+ cells during the 14 days of ex vivo expansion untreated or treated with 5-aza-2′-deoxycytidine. **a** Quantitative real time polymerase chain reaction for Chk1 using the Taq Man assay in mRNA extracts of samples collected every 3 days starting from the UCB CD133+ cells freshly isolated (day 0) up to day 14 from the ex vivo expansion and the mRNA of the terminally differentiated cells (diff.). Samples treated with 5-aza-2′-deoxycytidine at a dose of 0.05 μM as described in the text have also been included. Chk1 expression levels are normalized to the internal mRNA levels of the housekeeping control actin. The increase over the sample collected at time 0 is reported. **b** Western blot analysis showing Chk1, Chk2 and actin protein levels in samples collected at the indicated days during the ex vivo expansion as explained in Sect. "Materials and methods". Samples 9+, 12+ and 14+ represent protein extracts obtained from cells treated with 5-aza-2′-deoxycytidine

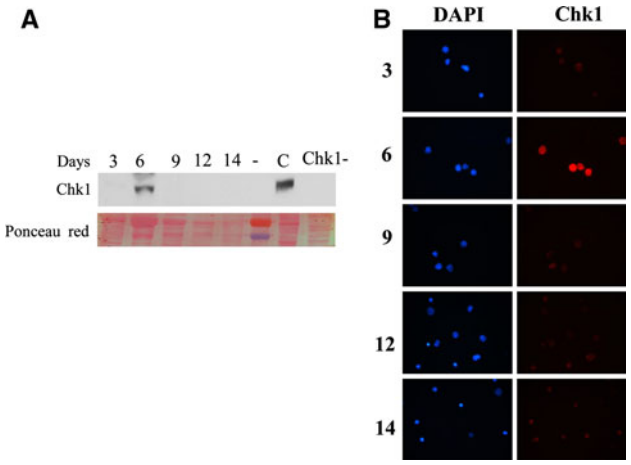

Fig. 4 Kinetics of Chk1 expression in CD133+ cells isolated from peripheral blood (PB) apheresis collected in healthy donors, after 3 days of G-CSF treatment. **a** Western blot analysis showing Chk1 protein levels in samples collected every 3 days starting from CD133+ cells isolated from peripheral blood (PB) by apheresis (from day 3 of the first induction in the patient up to day 14 of the ex vivo expansion with the cytokine cocktail). **b** Immuno-fluorescence analysis was also performed on these samples confirming the results obtained by Western blot analysis

was the same with a temporal shift, and in fact Chk1 level peaked at 6 days instead of 3 days from the beginning of induction. This probably reflects the different origin of CD133+ cells and the stimulation protocol used. Immunofluorescence confirmed the results of Western blot analysis (Fig. 4b).

Stem cells from UCB as a new "non-artificial" system expressing very low levels of Chk1

No normal somatic cellular systems with undetectable Chk1 protein levels have been described. Chk1 levels are cell-cycle phase specific and peak in S-phase [22], but in this system Chk1 levels seemed unlikely to be related to the S cycling fraction as at all time points cells were proliferating, as suggested by the growth curves (Suppl. Fig. 1). Interfering with Chk1 activity, by small interfering RNA (siRNA) and small molecules inhibiting its catalytic activity, has different effects on cell growth and cell morphology depending on the cells genetic background [4, 5, 23]. In our experimental system, Chk1 levels did not seem to correlate with cell growth or aberrant morphology. The cells grew apparently normally in this time frame (from day 3 to day 14) and DAPI staining showed normal cell morphology (Fig. 2c). Inhibition of Chk1 sensitizes tumor cells to DNA damaging agents and this effect is more pronounced in p53-deficient or mutated cells [23, 24]. In order to check whether high Chk1 protein levels 3 days after the ex vivo expansion led to different sensitivity to IR, UCB CD133+ cells were treated with IR at the time of purification (day 0) and after 3 and 14 days of ex vivo expansion. As shown in Suppl. Fig. 2, sensitivity to IR was comparable at the time points analyzed. These data suggest that in this experimental system Chk1 is not essential for growth and for the cytotoxic response to IR.

Functional explanation of the kinetics of Chk1 expression

In order to find a functional explanation for the unusual kinetics of Chk1 expression, we tried to downregulate Chk1 expression in UCB CD133+ ex vivo expanded cells on day 3 after starting expansion. This is the only time point when Chk1 is clearly detectable and (probably) functionally active. To downregulate Chk1 we used specific and effective siRNAs [23]. Different commercially available transfection reagents were used but unfortunately none was sufficiently efficient in transfecting CD133+ stem cells. A specific Chk1 inhibitor, CHIR-124, was then used [20].

To check whether the inhibitor interfered with Chk1 kinase activity, protein extracts were collected on day 3 from control and treated cells to detect the phosphorylation of Cdc25C phosphatase, a downstream Chk1 target, in ser 216 [25]. Phosphorylation appeared lower in cells treated with the Chk1 inhibitor than in the control cells, suggesting that Chk1 kinase activity was inhibited (Suppl. Fig. 3). Recently an additional marker of Chk1 inhibition has been reported i.e. its phosphorylation in ser317 and/or ser345 residues [26]. Indeed, we could detect an increase in serine 317 phosphorylation of Chk1 after treatment with CHIR-124. Cells were maintained in the presence of the drug till the end of the ex vivo expansion (14 days) when they were counted and characterized by flow cytometry for specific markers of stemness, myeloid, lymphoid and megakaryocytic differentiation. Table 1 shows the percentages of these markers in control and treated cultures (three independent experiments).

Table 1 Percentage of positive cells for the indicated antigens

Antigen	Experiment # 1		Experiment # 2		Experiment # 3	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
CD34	8	4.2	6.9	3.2	14	6.4
CD133	0.4	1	1	0.3	0.2	0.1
CD45	61.4	55	68.1	81	72.4	61.7
CD56	1.6	1.4	18.8	45.8	1.6	2
CD3	0	0	1.2	8.6	0.1	1
CD61	4.4	2	0.3	1	1	0.2
CD19	7.8	5.3	2.1	0.2	1.4	0.3
CD33	38.3	0.1	73	0.5	29.6	4.3
CD38	25.4	53.1	3.6	35.1	27.3	55.6

Results represent the relative proportion of cells positive for the differentiation markers selected both in untreated and in Chk1 inhibitor treated cells at day 14 of the ex vivo expansion. The table summarizes the results obtained in three independent experiments

As expected, CD133+ cells decreased in percentage but not in absolute number due to the commitment of some precursors and to the maintenance of the more primitive compartment (data not shown). Interestingly, we observed a different cell distribution based on the antigenic expression by flow cytometry in the samples treated with Chk1 inhibitor. In fact, the myeloid compartment characterized by the expression of the marker CD33 decreased and in parallel a higher expression of the percentage of CD38+ cells was detected. No co-expression of CD38 and CD33 was observed in both the cell populations (Fig. 5). Similar results were also obtained by treating the cells with another Chk1 inhibitor (AZD-7762) at the 3.75 nM, corresponding to its IC_{50} in this experimental system (Suppl. Fig. 4, panel a) [21]. To further investigate the possibility that Chk1 may be involved in the differentiation program of myeloid precursors, the myeloid leukemia cell line HL60 was treated with the two Chk1 inhibitors for 72 h (at the IC_{50} doses for this cell line) and a reduction in the CD33 marker expression was observed in HL60 treated cells as compared with untreated cells (Suppl. Fig. 4, panel b).

Discussion

Cells orchestrate a coordinated response to DNA damage, leading to cell cycle arrest, activation of DNA repair, or apoptosis if the damage is irreparable. The key roles of the activation of the DNA damage checkpoint pathways and the cellular DNA repair activities have been clearly demonstrated [2, 27]. While specific defects or up-regulation in these pathways are at the basis of tumor sensitivity or resistance to the cytotoxic effects of different anticancer agents, the same mechanisms are likely to be responsible for the myelotoxic effects of radio-chemotherapy. The

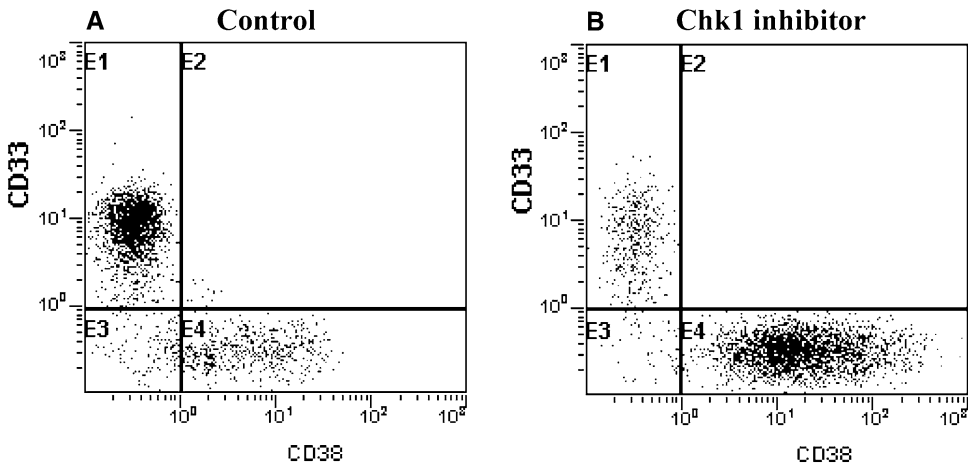

Fig. 5 Effect of Chk1 inhibition on the ex vivo expansion of cord blood progenitors cells after two week culture. **a** Representative dot plot of the control cultures where CD33 marker (myeloid differentiation) is highly expressed; **b** while in the cultures treated with the Chk1 inhibitor the CD38+ cells (lymphoid differentiation) are dramatically increased

sensitivity of the different HSPCs, derived from UCB, BM or peripheral blood, and their progeny to several anticancer agents has been analyzed in more detail in the last few years being now possible to isolate specific cell populations by phenotyping and sorting procedures [28]. However, the knowledge of the activation of DNA damage transduction pathways in hematopoeitic stem or progenitor cells is still poorly understood.

We isolated CD133+ cells from UCB and we cultured them with a specific cytokine combination to amplify the early progenitors and the more committed hematopoietic cell populations. This is an useful model for studying DNA damage checkpoint activation in cells with different degrees of staminality. The CD133+ cells and their progeny had similar sensitivity to IR. This finding differs from the CD34+ progenitor cells derived from BM and UCB which were less efficient at removing DNA lesions induced by EtNU, melphalan and cisplatin, than their mature CD34− counterparts; this lower DNA repair capacity was associated with higher induction of apoptosis. In contrast CD34+ cells from human BM were more efficient at nucleotide excision repair (NER) after UV damage than more mature CD34− cells [29, 30]. These contrasting results may be due to differences in the experimental systems. Interestingly, we found that the negative fraction obtained after CD133+ isolation, corresponding to the whole mature UCB cells, was indeed very resistant to the same IR, possibly because these are non-dividing terminally differentiated cells against which no DNA damaging agents would have any effect. The DNA damage checkpoint activation after IR was as in other cellular systems: p53 stabilization after IR was found from 1 h up to 24 h in both the CD133+ cells and the ex vivo expanded progeny, and Chk2 was normally expressed and slightly activated. These markers are usually activated after IR: p53 is almost undetectable in normal non-stressed conditions, but is rapidly activated in response to DNA damage and has a key role in the G_1 checkpoint and in sustaining a G_2–M block [31]; Chk2 is a checkpoint kinase activated by ATM upon IR that can phosphorylate p53 and cdc25 phosphatases to induce cell cycle arrest and favor repair [32]. These data clearly demonstrate that CD133+ and the more committed cells derived from them respond similarly to IR induced damage. Studies are in progress to verify if this is true for other DNA damaging agents. On the contrary they differ in Chk1 expression. In fact, when we analyzed Chk1, having a key role in the S, G_2M and in the mitotic spindle checkpoint [33–35], the protein was undetectable in the ex vivo expanded population derived from UCB CD133+ cells. This result was intriguing and somehow paradoxical. Chk1 is indispensable for mammalian survival. Chk1−/− mice die at E6.5 days indicating that it has a fundamental role in early embryonic development [36]. The conditional KO of Chk1 caused apoptosis in mice embryonic stem cells and led to premature mitosis before completion of DNA replication and cell death by mitotic catastrophe [37]. Chk1 is essential for the growth of mammary proliferating epithelial cells [6]. Evidence on Chk1's role in somatic cancer cell lines is, however, controversial. The only available vertebrate Chk1 KO somatic cancer cellular system, an avian lymphoma cell line, is viable but has multiple checkpoint and survival defects [38]. Chk1 depletion by siRNA did not alter the cell cycle profile or induce apoptosis in the absence of DNA damage in many other different tumor systems, though our group recently reported that U2OS (an osteosarcoma cell line) cells with Chk1 depleted die, suggesting that Chk1 is essential here [5, 23].

The data on the lack of Chk1 expression in UCB CD133+ cells ex vivo expanded are, to our knowledge, the first indicating that Chk1 is not essential for the survival of normal human proliferating cells. In addition, we found that Chk1 expression followed a precise kinetics of induction during the ex vivo expansion, being almost absent in the freshly isolated CD133+ cells, present at day 3 of the cytokine induction and disappearing thereafter. Chk1 levels were not related to the cell proliferation status since cells were cycling at all the different time points, except maybe at time 0 when they were freshly isolated and mostly in G_0–G_1 phases (data not shown). The expression is transcriptionally regulated, as shown by the RT–PCR data, and does not seem to depend on the Chk1 promoter methylation as 5-aza-2′-deoxycytidine did not reverse the protein expression. This peculiarity was corroborated in a different experimental system in which CD133+ cells were isolated from PB by apheresis in a patient treated with G-CSF for 3 days, the time necessary to mobilize HSPCs to the blood. When these cells were cultured with the same cytokine cocktail they showed the same Chk1 expression profile, suggesting that Chk1 might have a role in hematopoietic differentiation.

This pattern of Chk1 expression does not correlate with any difference in sensitivity to IR, unlike many other experimental tumor systems, clearly showing that the lack of Chk1 conferred higher sensitivity to IR treatment [23, 38]. To understand the specific temporal expression of Chk1 during the ex vivo expansion of CD133+ cells, we treated freshly isolated UCB CD133+ cells with a specific Chk1 inhibitor (CHIR-124), and we observed a clear modification in the differentiation pathway. In fact, CD133+ cells treated with the inhibitor showed a striking and significant decrease in myeloid commitment (Table 1) with a much lower expression of the marker CD33, which identifies various stages of myeloid cell differentiation. In addition, CD61, the glycoprotein IIIa, expressed on cells of the megakaryocytic lineage, was slightly reduced in the treated cells. Interestingly, the Chk1-inhibited cells showed

a higher expression of the marker CD38 that is usually expressed by itself on lymphoid precursors and on early hematopoietic progenitors if the cells are also negative for standard lineage markers. Otherwise CD38 can be expressed in association with the marker CD33 to define immature myeloid precursors. As shown in Fig. 5b, in our experiments no co-expression of CD38 and CD33 was observed indicating a possible lymphoid/myeloid shift of the CD133+ cells during the expansion. Similar results were obtained with another Chk1 inhibitor (AZD 7762), corroborating the fact that in this experimental setting the temporal pattern of Chk1 expression during expansion may be related to a possible Chk1 role during the cell commitment and differentiation. The decrease of expression of CD33 marker in the myeloid leukemia cell line HL-60 after treatment with the two Chk1 inhibitors, strengthened these data. This putative role of Chk1 in hematopoietic differentiation is worth considering in relation to the recent observation that Chk1 is a histone H3 kinase, responsible for DNA-damage-induced transcriptional repression by loss of histone acetylation. Specifically, phosphorylation of histone H3-threonine 11 (H3-T11) was recently identified as a novel chromatin modification for transcriptional activation, rapidly reduced after DNA damage [7]. Chk1 binds chromatin and phosphorylates H3-T11 in unperturbed physiological conditions. After DNA damage Chk1 dissociates from chromatin and this closely correlates with the decrease in phosphorylation of H3-T11 and in the binding of GCN5, a family of histone acetylases, with H3, leading to reduced H3-K9 acetylation and subsequent repression of transcription of target genes. Chk1-dependent transcriptional repression has been described after DNA damage, and it might be that this specific H3-T11 phosphorylation and possibly other core histone phosphorylations mediated by Chk1 occur also in unperturbed conditions, interfering with the normal transcriptional machinery, including different commitment programs, such as hematopoietic differentiation. A role for Chk1 in T lineage differentiation in thymocytes has recently been described, suggesting a possible involvement of this protein in the differentiation program of lymphoid lineage [39]. The finding that in this experimental system inhibition of Chk1 is related to a cell differentiation shift in UCB CD133+ hematopoietic stem cells puts Chk1 at a key cross-road where many biological processes meet (cell cycle progression, checkpoint function, DNA repair and transcription). The definition of Chk1's different roles is important because Chk1 inhibitors are being developed in Phase I clinical trials for use in combination with anticancer agents.

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