Doxorubicin induces the DNA damage response in cultured human mesenchymal stem cells.

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Running title: hMSCs sensitivity to doxorubicin.
Abstract

Anthracyclines including doxorubicin are widely used in the treatment of leukemia. While the effects of doxorubicin on hematopoietic cells have been characterized, less is known about the response of human mesenchymal stem cells (hMSCs) in the bone marrow stroma to anthracyclines. We characterized the effect of doxorubicin on key DNA damage responses in hMSCs, and compared doxorubicin sensitivity and DNA damage response activation between isolated hMSCs and the chronic myelogenous leukemia (CML) cell line, K562.

Phosphorylation of H2AX, Chk1 and RPA2 was more strongly activated in K562 cells than in hMSCs, at equivalent doses of doxorubicin. hMSCs were relatively resistant to doxorubicin such that following exposure to 15μM doxorubicin, the level of cleaved caspase-3 detected by western blotting was lower in hMSCs compared to K562 cells. Flow cytometric analysis of cell cycle progression demonstrated that exposure to doxorubicin induced G2/M phase arrest in hMSCs, while, 48 hours after exposure, 17% of cells were apoptotic as determined from the percentage of cells having sub-G1 DNA content. We also show that the doxorubicin sensitivity of hMSCs isolated from a healthy donor was comparable to that of hMSCs isolated from a chronic lymphocytic leukemia (CLL) patient. Overall, our results demonstrate that high doses of doxorubicin induce the DNA damage response in hMSCs, and that cultured hMSCs are relatively resistant to doxorubicin.
**Introduction**

Mesenchymal stem cells (MSCs), also referred to as bone marrow stromal cells or mesenchymal progenitor cells [1, 2] constitute a small but critical fraction of the total population of nucleated cells in bone marrow [3]. MSCs are progenitors of cells of mesenchymal origin, including osteoblasts, chondrocytes and adipocytes, and also play a crucial role in maintaining the normal function of hematopoietic stem cells (HSCs) within the bone marrow microenvironment [4]. Agents that affect MSC number or function can therefore directly affect tissue homeostasis, and indirectly modulate the behavior of HSCs, with implications for development and treatment of hematological malignancies [5].

The anthracycline doxorubicin is one of the most effective anticancer drugs, and exhibits activity against a wide spectrum of solid tumors, lymphomas, and leukemia [6]. The use of doxorubicin is however limited by the associated cardiomyopathy [6]. Despite extensive clinical use, the mechanism of action of anthracyclines in cancer cells is still under investigation. Doxorubicin has been proposed to exert its cytotoxic effect through a number of mechanisms, including i) inhibition of topoisomerase II, resulting in the formation of DNA double-strand breaks, ii) generation of oxygen free radicals, and iii) formation of intercalating doxorubicin DNA adducts that prevent DNA replication [6, 7]. Overall, DNA damage is key to doxorubicin-induced cell death following treatment [6]. An orchestrated signaling cascade termed the DNA damage response (DDR) mediates the cellular response to damage, including cell cycle arrest, DNA repair and induction of apoptosis [8, 9]. Both single-stranded DNA generated by replication fork arrest, and DNA strand breaks resulting directly from DNA damage or indirectly from replication fork collapse, activate downstream DDR pathways mediated by the phosphoinositide 3-kinase (PI-3K)-related protein kinases (PIK kinases). PIK kinase-dependent phosphorylation of an array of downstream transducer and effector proteins, including, for example, histone H2AX and replication protein A (RPA), plays major role in determining the outcome of exposure to DNA damaging agents [9, 10].

A better understanding of the response of hMSCs to doxorubicin could provide new insights into the effects of this anticancer drug. Reduced MSC number or function in the bone marrow following exposure to doxorubicin could have an impact on normal hematopoietic stem cell function [4, 11, 12], while MSCs that survive DNA damage could contribute to secondary cancer development. Doxorubicin-induced loss of MSCs could also be significant in the context of doxorubicin-induced cardiotoxicity, as this may reduce the pool of MSCs available for differentiation towards the cardiomyocyte lineage [13].

We have previously reported that, when compared to peripheral blood lymphocytes or the CML cell line K562, both normal hMSCs and hMSCs derived from a CLL patient were more resistant to cisplatin and γ-irradiation, used in treatment of many solid tumors [14]. Since MSCs can be exposed to doxorubicin during the treatment of leukemia [15], the aim of this study was to determine the activation of key DNA damage responses in hMSCs exposed to doxorubicin, and to compare the sensitivity of hMSCs to that of the leukemia cell line K562. The results provide new insights into the effects of doxorubicin on hMSCs, a critical stem cell population in the bone marrow.
Materials and Methods

Cell isolation and treatment
Bone marrow aspirates were obtained, and hMSCs were characterized as previously described [14]. Prior to treatment with doxorubicin, hMSCs were passaged up to five times. Briefly, 8x10^4 cells were seeded in 100 mm dishes in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories) and 1% penicillin/streptomycin. Cells were either mock-treated, or treated with doxorubicin 48h after seeding [14]. Cells were routinely treated with either 15μM or 18.4μM (10μg/ml) doxorubicin as indicated in individual experiments. Data are representative of results obtained using hMSCs isolated from the bone marrow of at least three normal donors. hMSCs were isolated from one CLL patient, as previously described [14]. K562 human chronic myelogenous leukemia (CML) cells were cultured in RPMI 1640 medium as described previously [14]. 24 h prior to treatment, cells were seeded at 2x10^5 cells/ml in 100mm dishes, and were then treated with doxorubicin as indicated for individual experiments. Control cells were treated with an equivalent volume of water.

Immunoblotting
Cells were washed in PBS, and lysed in RIPA buffer (NaCl 150mM, 50mM Tris HCl pH7.4, 1% NP40, 0.25% DOC, 1mM EDTA) containing 1 mM Na3VO4, 5 mM NaF, 1 mM PMSF, 2 μg/ml aprotinin and 1 μg/ml leupeptin. Protein concentration was determined using the DC protein assay (BioRad). Proteins were separated by 12% SDS-PAGE, and transferred to PVDF membrane. Membranes were incubated overnight at 4°C with the appropriate antibodies, and bound antibody was detected as previously described [14]. Western blots were carried out at least twice on extracts of cells from each donor.

Flow cytometry
Following treatment with doxorubicin, cell cycle progression was analyzed by flow cytometry [16] using a FACS Calibur or a FACSCanto II instrument. Data was analyzed using CellQuest™ software, and Diva™ software. Cell cycle distribution was determined from the cycling population only, excluding cells with sub-G1 DNA content from the analysis. Induction of cell death was calculated from the percentage of sub-G1 cells in the total cell population.

Cell viability
hMSCs from healthy donors or from a CLL patient were seeded in triplicate wells in 96-well flat-bottom plates, at 5 x 10^3 cells per well. Cells were either mock-treated or treated with doxorubicin at the indicted doses. Cell viability was determined using the XTT assay, 48 hours after drug treatment [14, 16].
Results and Discussion

**Doxorubicin induces the DNA damage response and cell cycle arrest in hMSCs.**

Doxorubicin-induced DNA damage underlies the cytotoxic effects of this drug [6]. A network of sensor, mediator, transducer and effector proteins mediates the cellular response to DNA damage [8]. We used western blotting to directly compare the effects of doxorubicin on a number of DNA damage response (DDR) proteins in hMSCs isolated from healthy donors and in K562 cells, a widely used model leukemia cell line (Figure 1). In the absence of doxorubicin exposure, the DDR was not strongly activated in cultured hMSCs or in K562 cells (Figure 1, compare hMSC and K562 panels). The level of the tumor suppressor p53 protein [17] increased in hMSCs after doxorubicin treatment, consistent with p53 stabilization following doxorubicin-induced DNA damage [18, 19], and as previously observed in response to treatment of hMSCs with cisplatin [14, 20, 21]. However, consistent with the p53-null status of K562 cells due to a frameshift mutation in the TP53 gene [22], p53 was not detected in K562 cell lysates (Figure 1B).

To determine whether doxorubicin activated the S and G2 phase cell cycle checkpoints [23], the status of Chk1, a key regulator of these checkpoints, was examined [19]. In response to DNA damage, Chk1 regulates S-phase progression by phosphorylation and inactivation of Cdc25A, and mitotic progression by preventing the activation of Cdk1/cyclin B. Chk1 also plays a crucial role in the induction of apoptosis, and is a target for caspase-mediated cleavage [19, 24]. As shown in Figure 1, following exposure of hMSCs to 15μM doxorubicin, Chk1 was phosphorylated on serine 317. Treatment of K562 cells with 15μM doxorubicin led to a strong induction of Chk1 phosphorylation, as well as the appearance of a phosphorylated and cleaved form of Chk1, 24h and 48h following treatment. Phosphorylation of Chk1 was also strongly induced as early as 6h following treatment of K562 cells with 1μM doxorubicin (supplementary Figure 1). However, the extent of phosphorylation was much lower in hMSCs after treatment with 15μM doxorubicin compared to in K562 cells exposed to either 15μM (Figure 1) or 1μM doxorubicin (Supplementary Figure 1). The decrease in the total level of Chk1 at later times post-treatment is coincident with the appearance of the cleaved form of Chk1, consistent with strong induction of apoptosis especially in K562 cells under these conditions (see Figure 1, cleaved caspase-3 panels). Chk1 is known to be cleaved in cells undergoing apoptosis [25, 26] and the appearance of cleaved Chk1 and the reduction in total Chk1 levels in doxorubicin-treated K562 cells is consistent with induction of apoptosis under these conditions.

To characterize doxorubicin-induced activation of the apoptotic pathway in hMSCs, caspase-3 cleavage was analyzed [18] by western blotting. As shown in Figure 1A, treatment of hMSCs with 15μM doxorubicin induced slight cleavage of caspase-3, 48h after treatment. Direct comparison of the level of caspase-3 cleavage between K562 cells and hMSCs after treatment with 15μM doxorubicin, showed very strong caspase-3 cleavage after 24h and 48h in K562 cells (Figure 1B), compared to in hMSCs (Fig. 1B, right lane). Strong cleavage of caspase-3 was also readily observed in K562 cells, 48h after treatment with 1μM doxorubicin (supplementary Figure 1). These results indicate that compared to K562 cells, hMSCs are relatively resistant to doxorubicin-induced apoptosis, as determined by caspase-3 cleavage, (Figure 1).

Doxorubicin-induced DNA damage can result in DNA strand breaks, ultimately leading to apoptosis [6, 27].
PIK kinase-dependent phosphorylation of downstream transducer and effector proteins, including histone H2AX and replication protein A (RPA), plays a major role in determining the cellular response to DNA strand breaks [9, 10]. Human histone H2AX is phosphorylated on serine 139, generating $\gamma$-H2AX, which plays a key role in recruitment of repair proteins to strand breaks [28, 29]. $\gamma$-H2AX was slightly increased 48h after treatment of hMSCs with 15μM doxorubicin (Figure 1). $\gamma$-H2AX was increased 24h after treatment of K562 cells with 15μM doxorubicin, while the level decreased after 48h, probably due to strong induction of apoptosis (Figure 1). This response was also induced after treatment of K562 cells with 1μM doxorubicin, with a high level of $\gamma$-H2AX detected 48h after treatment (supplementary Figure 1). RPA2, the 32kDa subunit of replication protein A (RPA), the major human single-stranded DNA binding protein in human cells with roles in all aspects of DNA metabolism [30] is also phosphorylated in a DNA damage-dependent manner on serine 4 and serine 8 [16]. Doxorubicin treatment induced phosphorylation of RPA2 both in hMSCs and in K562 cells, but the level of RPA2 phosphorylation was higher in K562 cells compared to hMSCs (Figure 1), indicating stronger DDR activation in K562 cells.

Effect of doxorubicin on cell cycle progression in hMSCs.
Since doxorubicin induced phosphorylation of Chk1 protein in hMSCs (Figure 1), the effect of doxorubicin on cell cycle progression in normal hMSCs was analyzed using propidium iodide staining and flow cytometry. In untreated hMSCs, the percentage of cells in the G0/G1 phase of the cell cycle increased with time, while the proportion of cells in S phase decreased (Figure 2A, 2B). However, treatment of hMSCs with 18.4μM doxorubicin for 48h led to a decrease in the percentage of cells in G0/G1, from 82.1±3.0 % in control cells to 60.5±2.5% in doxorubicin-treated cells (Figure 2B). The decrease in G0/G1 cells was associated with a decrease in the percentage of cells in S phase (from 8.5±2.1% in control cells to 3.5±1.7% in doxorubicin-treated cells), and an increase in the percentage of cells in the G2/M phases of the cell cycle (from 9.4±1.0% in control cells, to 18.3±1.8% in doxorubicin-treated cells) (Figure 2B). The differences between control and doxorubicin-treated cells are statistically significant for the percentage of cells in S phase and in the G2/M phases (S phase: p < 0.01 at 24h and p < 0.05 at 48h; G2/M phase: p<0.001 at 24h, and p< 0.01 at 48h). These results are consistent with doxorubicin-induced S-phase arrest, due to the formation of DNA adducts that prevent DNA replication [7], and G2 checkpoint activation that may result from double-strand break formation downstream of topoisomerase II inhibition [6]. While doxorubicin-induced cell cycle arrest in the S and G2/M phases, as well as induction of cell death, has been demonstrated in murine lymphocytes [31] and in the promyelocytic leukemic cell line HL-60 [7], this is the first demonstration that doxorubicin induces cell cycle arrest in hMSCs. To determine the extent of doxorubicin-induced cell death in hMSCs, the percentage of cells having sub-G1 DNA content was also analysed by flow cytometry (Fig. 2C). Following exposure of hMSCs to doxorubicin for 48h, 17.0±1.9% of the cells were in the sub-G1 population. As shown in supplementary Figure 1B and1C, treatment of K562 cells with doxorubicin for 48h also induced apoptosis; exposure of cells to 1μM doxorubicin resulted in 62.6±4.2% of K562 cells being in the sub-G1 fraction.

Doxorubicin-induced apoptosis can result from up-regulation of Fas expression, and activation of the classic mode of apoptosis involving enhanced caspase activity to promote intracellular apoptotic signaling [6, 7, 31]. Overall, our results showing that cultured hMSCs are relatively resistant to doxorubicin-induced apoptosis are consistent with the report that a proportion of mesenchymal progenitor cells in bone marrow can survive COSS-
96 polychemotherapy, including doxorubicin, methotrexate, cisplatin and ifosfamid [32]. Given that anthracyclines are widely used in the treatment of leukemia, resistance of hMSCs to doxorubicin treatment could be relevant to the development of secondary cancers such as sarcomas [33,34].

**hMSC viability following exposure to doxorubicin.** We have previously compared DNA damage responses in hMSCs from healthy donors and from a patient with chronic lymphocytic leukemia (CLL), after exposure to the chemotherapeutic drug cisplatin or to ionizing radiation [14]. We did not observe differences in DDR activation or cell viability between hMSCs from healthy donors and cells from the CLL patient, in response to these two agents [14]. We have also previously shown that, compared to PBLCs or K562 cells, hMSCs from both healthy donors and from the CLL patient are relatively resistant to cisplatin and ionizing radiation [14]. To determine the doxorubicin sensitivity of hMSCs from a healthy donor and CLL-derived hMSCs, isolated hMSCs were treated with doxorubicin for 48h and cell viability was determined using the XTT assay (Figure 3). For both healthy and CLL-patient-derived hMSCs, cell viability decreased in a dose-dependent manner, and no significant difference was observed between the doxorubicin-sensitivity of hMSCs from the two sources.

Overall, the present data show that exposure of hMSCs to doxorubicin activates key DNA damage response pathways, consistent with studies of other stem cell types including HSCs [35, 36] and embryonic stem cells [37, 38]. While doxorubicin activates the DNA damage response and cell cycle checkpoints, hMSCs are relatively resistant to the cytotoxic effects. This is consistent with our previous demonstration that hMSCs both from healthy donors and from a patient with CLL are resistant to cisplatin and ionizing radiation [14]. The relative resistance of hMSCs to DNA damaging agents used in cancer therapy (this study, [14, 15]), is also relevant to solid tumours, as it has been reported that relocation of murine MSCs from the bone marrow creates a niche that sustains cancer progression [39]. The recent demonstration that DNA damage-induced secretion of paracrine factors by MSCs can increase the resistance of tumor cells to cell killing also highlights the potential importance of MSCs in the response to cancer treatment [40].

**List of abbreviations:** hMSC, human mesenchymal stem cells; DDR, DNA damage response; ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; DNA-PK, DNA-dependent protein kinase; Chk1, checkpoint kinase 1.

**Competing interests**
The author(s) declare that they have no competing interests

**Authors contributions**
SCH, AMP, FB and MPC conceptualized and designed the study. GS isolated and characterized hMSCs. SCH performed experiments and analysed data comparing hMSCs and K562 cells, and healthy versus CLL-derived hMSCs. AMP and SCH performed experiments and analysed data on cell cycle progression in hMSCs, and induction of sub-G1 cells. SCH and MPC wrote the paper. All authors read and approved the final manuscript.
Acknowledgements

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References

Figure Legends

Figure 1: Doxorubicin-induced DNA damage responses in hMSCs and K562 cells. hMSCs (left panels) were treated with 15μM doxorubicin for the indicated times. DNA damage response proteins were analyzed by western blotting, as described in Materials and Methods. K562 cells (right panels) were treated with 15μM doxorubicin for the indicated times. DNA damage response proteins were analyzed by western blotting. For direct comparison, western blots of extracts derived from hMSCs treated with 15μM doxorubicin for 24h (for phosphoSer4/Ser8 RPA2, phosphoSer317-Chk1, Chk1, γ-H2AX, and cleaved caspase 3, or for 48h (for p53, and total RPA2), and run on the same gels as the K562 cell extracts, are shown (hMSC + doxorubicin, right lane). Numbers indicate the position of molecular size markers (kDa).

Figure 2: Flow cytometric analysis of cell cycle progression in hMSCs treated with doxorubicin. A. Flow cytometry histograms from a representative experiment for the determination of cell cycle distribution in mock-treated (control) hMSCs or cells treated with 18.4μM doxorubicin. DNA was stained with propidium iodide (PI), and DNA content was analyzed by flow cytometry. B. The percentage of cells in the G0/G1, S and G2/M phases was determined for control cells or cells treated with doxorubicin after staining DNA with propidium iodide as described in A. Data is the mean of three independent experiments; error bars represent one standard deviation. The percentage of cells in the G0/G1 and the G2/M phases is significantly different (p <0.001) between untreated cells and doxorubicin-treated cells, as determined using two-way Anova analysis. C. The percentage of sub-G1 cells was determined for cells treated with doxorubicin, after staining DNA with propidium iodide as in A. Data is the mean of two independent experiments; error bars represent one standard deviation.

Figure 3: Sensitivity of hMSCs derived from a healthy donor or a CLL patient to doxorubicin. Cells were treated with the indicated doses of doxorubicin for 48 hours. Cell viability was determined using the XTT assay, and is expressed as a percentage of the viability of untreated cells. Data represent the mean of three independent experiments; error bars represent one standard deviation.

Supplementary Figure 1: DNA damage responses and apoptosis induction in K562 cells treated with 1μM doxorubicin. A. K562 cells were treated with 1μM doxorubicin for the indicated times. DNA damage response proteins were analyzed by western blotting, as described in the legend to Figure 1, and in Materials and Methods. B. Flow cytometry histograms from a representative experiment, showing the induction of sub-G1 cells, 48h after mock-treatment or treatment of K562 cells with 1μM doxorubicin. C. The percentage of sub-G1 K562 cells was determined following propidium iodide staining and flow cytometry, 48h after treatment. Data represent the mean of two independent experiments; error bars represent one standard deviation.
Figure 1

hMSCs

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K562 cells

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**Abbreviations:**
- p53
- Phospho-Ser317-Chk1
- Total Chk1
- Cleaved caspase-3
- γ-H2AX
- Phospho-Ser4/Ser8-RPA2
- RPA2
- Actin

**Legend:**
- NS: Not significant
- p-Ser317-Chk1
- Cleaved Chk1
- Chk1
- Cleaved Chk1
- p-Ser317-Chk1
- Cleaved Chk1
- Chk1
- Cleaved Chk1
- RPA2-PP
- RPA2
Figure 2

A) Time (hours) 0 6 24 48

Untreated

Doxorubicin

Counts

DNA

B) Cell cycle distribution after doxorubicin treatment

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G0/G1 S G2/M

C) SubG1 population after doxorubicin treatment

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Sensitivity of hMSCs to Doxorubicin

- **Figure 3**

- Sensitivity of hMSCs to Doxorubicin

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Legend:
- Healthy donor
- CLL donor
Supplementary figure 1

K562

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- **NS p53**
- **PhosphoSer317-Chk1**
- **Cleaved Chk1**
- **Chk1**
- **Cleaved Chk1**

- **Cleaved caspase-3**
  - Long exposure
- **Cleaved caspase-3**
  - Short exposure
- **γ-H2AX**
- **Phospho-Ser4/Ser8-RPA2**
- **RPA2-PP**
- **RPA2**
- **Actin**

B) **Control**

Doxorubicin

DNA

Counts

C) **K562 cells treated with doxorubicin**

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