Cytosine Arabinoside reduces the numbers of granulocyte macrophage colony forming cells (GM-CFC) and high proliferative potential colony forming cells (HPP-CFC) in vivo in mice

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Abstract

Background: Cytosine arabinoside (Ara-C) is an S-phase specific cytotoxic drug used in the treatment of malignancies. It is converted to Cytosine Arabinoside triphosphate (Ara-CTP) in the cell. Cytosine Arabinoside triphosphate, reversibly displaces deoxy cytidine triphosphate from DNA polymerase for incorporation into DNA. This process leads to cell death.

Objective: To investigate the in vivo effects of Ara-C on the Granulocyte Macrophage Colony Forming Cells (GM-CFC) and High Proliferative Potential Colony Forming Cells (HPP-CFC) respectively in mice.

Methodology: Ara-C (150mg/kg) was administered intraperitoneally (i.p) once to mice and bone marrow cells sampled on days 1, 3 and 6.

Results: Ara-C reduced the numbers of both GM-CFC and HPP-CFC in the bone marrow. HPP-CFCs were initially more sensitive to Ara-C treatment than GM-CFCs. In the six days after treatment the effect on GM-CFC persisted, while there was a partial recovery in the number of HPP-CFCs.

Conclusion: It is possible that Ara-C disturbs the stem cells niche by damaging the stromal cells of the bone marrow microenvironment. This would result in derangement of HPP-CFC proliferation.

Introduction

Cytosine arabinoside (Ara-C) is a drug used in the treatment of leukaemias and lymphomas, always in combination with other drugs. It works by interfering with pyrimidine synthesis in actively dividing cells, both normal and pathological. Better understanding of the way that Ara-C affects various cell lines in the bone marrow may help us to devise improved ways of deploying this drug in the management of neoplastic disease. In this study we have used a mouse model to analyse the differential effects of Ara-C on two important precursor cell lines in the bone marrow.

The haematopoietic system is composed of cells that are arranged in a hierarchy in terms of primitiveness as well as differentiation potential. The pluripotent stem cell population and their progeny are surrounded by stromal cells in the bone marrow. The haematopioetic stem cell (Long term-Haematopoietic Stem Cell) population is believed to be mostly in quiescent state but has a high self-renewal potential due to the bone marrow microenvironment that preserves this cell population in a specific niche. Above the stem cells pool are progenitor cells (Short term-Haematopoietic Progenitor Cell) that are believed to have a high population of cells in cell cycle[1,2] with a great number of them in a differentiated state. Therefore, the stem cell population can become vulnerable to differentiation pressures if the niche is damaged while the progenitor cell population is vulnerable due to a high rate of cell turn over. Ara-C is an S-phase specific cytotoxic drug and it has been shown to induce K562, a human erythroleukaemic cell line that has stem cell potential, into erythroid differentiation[3]. Once Ara-C enters the cell, it is converted to its active form Cytosine Arabinoside triphosphate. This active form reversibly displaces deoxy cytidine triphosphate from DNA polymerase for incorporation into DNA, a process that leads to formation of damaged and abnormal DNA[4,5]. Because Ara-C kills cells that are actively dividing – neoplastic as well as normal cells – one of the side effects of the drug is myelo-suppression resulting in granulocytopenia, anaemia and thrombocytopenia[6,7].

The bone marrow microenvironment consists of stromal cells that originate from mesenchymal stem cells[8]. In vitro studies have shown that mesenchymal stem cells produce a number of cytokines and extracellular matrix proteins and they also express cell adhesion molecules, all of which are involved in the regulation of haematopoiesis both in vivo and in vitro[9,10]. In vitro studies have shown that Ara-C may irreversibly damage the bone marrow microenvironment by reducing the viable mesenchymal stem cells by about 20% at clinically relevant concentrations[11]. It is possible that mesenchymal stem cells might become vulnerable together with haematopoietic cells following Ara-C administration. This could result in the reduced ability of stromal cells to sustain haematopoiesis during Ara-C administration.

The purpose of this study was to investigate the effects of Cytosine Arabinoside (150mg/kg) on the haematopoietic pluripotent stem cells which are normally in a quiescent state as compared to the progenitor cells that have a large pool of cells in cell cycle (Fig 1). We investigated changes in the numbers of these two cell lines in mice after days, 1, 3 and 6 following Ara-C (150mg/kg) drug administration.

Material and Methods

The bone marrow cells were plated in semi solid agar cultures according to established methods for assaying GM-CFC and HPP-CFC[12,13]. Previous work has shown that patients who receive the dose of 150mg/kg do not develop severe pancytopenia (14). All experiments were carried out using mice, regardless of sex and weighing an average of 25 g, purchased from Thondwe in Zomba from a local farmer after approval from the College of Medicine Research Ethics Committee (COMREC). In order to acclimatise the mice, they were kept in the laboratory for 2 weeks before experiments were conducted, and were looked after according to standard animal maintenance procedures. They were divided into two groups each consisting of 3 animals. One group was injected intraperitoneally with 150 mg/kg Ara-C dissolved in normal saline at day 0, and mice in the other group were injected with a similar volume of normal saline to serve as controls. One animal from each group was sacrificed (by cervical dislocation) at days 1, 3, and 6 after injection. No attempt was made to determine the number of nucleated bone marrow cells per femur. The percentage of HPP-CFC in S-phase was not investigated and is currently not known.

Granulocyte-Macrophage Colony-Forming Cell (GM-CFC) Assay

Using a fine needle, pooled bone marrow cells were flushed with Dulbecco's modified Eagle's medium (Sigma,UK) supplemented with 20% horse serum (HS), 50 IU benzyl penicillin, 50 μg/ml streptomycin and 2mM L-glutamine (D20% HS PS/G). A final concentration of 105 cells/ml was suspended in D20% HS PS/G with 0.3% melted agar (Bacto Agar, Difco Labs; Detroit, MI). 1ml of cell suspension was plated in each non-tissue culture grade 30-mm Petri dish (Sterilin; Stone, UK) in the presence of 0.1ml of L929 conditioned medium as a crude source of macrophage colony stimulating factor (M-CSF)(15). Petri dishes were incubated in the fully humidified air/10% CO2 incubator at 37O C (LEEC Ltd, UK) for seven days. At day six, the vital stain 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) (BDH Chemicals Ltd; Poole, UK) was added and stained colonies were counted macroscopically on day 7.

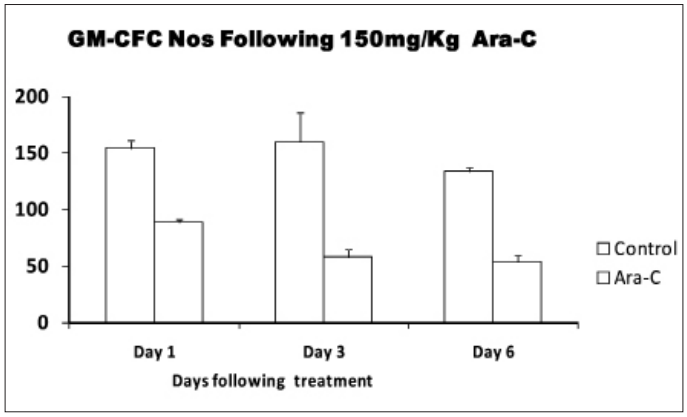
High Proliferative Potential Colony-Forming cell (HPP-CFC) Assay

Pooled bone marrow cells were grown over a feeder layer made up of 0.5% melted agar (Bacto Agar, Difco Labs) in D20% HS PS/G supplemented with WEHI-3B-and L929-conditioned media used as crude source of interleukin 3 and macrophage-colony stimulating factor. A cellular layer was made up of 105 nucleated cells/ml in D20% HS PS/G with 0.3% melted Agar. Cultures were incubated in a fully humidified air/10% CO2 incubator at 37O C. On day 13, INT was added and colonies (>2mm) were counted on day 14 macroscopically [12,13].

Statistical Analysis

Four independent experiments were performed for each group of experiments. In total twenty-four mice were used for the experiments. Eight petri dishes were plated for each batch for GM-CFC (32 for Ara-C and 32 for Saline in total) and four petri dishes were plated for each batch for HPP-CFC (16 for Ara-C and 16 for Saline in total). The statistical significance of the results was determined by the unpaired Students' t-test. A p-value of 0.05 or less was considered significant.

FIG. 1. Numbers of GM-CFC per 105 nucleated cells in the bone marrow of mice injected intraperitoneally with 150mg/kg body weight of Ara-C (shaded bars) or with a similar volume of normal saline (open bars) on different days after treatment. On each of the sampling days, the mean number of GM-CFC colonies was significantly less ($p<0.001$) in the Ara-C treated group than the saline-treated controls. The colony numbers in Ara-C treated mice are not increasing with time.



Results

The effect of Cytosine Arabinoside (Ara-c) on GM-CFC numbers in vivo.

As can be seen in Fig.1, on each of the sampling days there were fewer GM-CFC colonies in the petri dishes containing bone marrow cells from mice treated with Ara-C as compared to the mice treated with normal saline. On each day the difference in mean number of colonies between saline controls and Ara-C treated mice was statistically significant with p-values of less than 0.001. There was no change in the mean count of GM-CFC colonies between the three sampling days.

The effect of Cytosine Arabinoside on HPP-CFC numbers in vivo

As shown in Fig. 2, on day 1, only 4.0 ± 2.0 per 105 nucleated cells HPP-CFC colonies were grown from Ara-C treated mice compared to 108 ± 24.8 per 105 nucleated cells ($p < 0.0001$) from mice treated with normal saline. The mean number of colonies in Ara-C treated mice remained similar on day 3 but by day 6 had increased, although still significantly less than in the saline-treated group ($p<0.01$). The increase in mean HPP-CFC colony counts in Ara-C treated mice between day 1 and day 6 was highly significant ($p<0.0001$)

Comparison of the percentages change GM-CFC from and HPP-CFC following Cytosine Arabinoside (150mg/kg intraperitoneally) treatment relative to Normal Saline controls.

Fig.3 presents the same figures but expresses the mean colony counts in Ara-C treated mice as a percentage of the equivalent mean counts in saline-treated controls, for each of the three sampling days. There was a statistically significant difference in colonies numbers between GM-CFC and HPP-CFC colonies in the Ara-C treated group on days 1 and 3 ($p < 0.0001$), with much lower mean counts for HPP-CFC than for GM-CFC. On day 6 there was no statistical significant difference between the colony numbers of the two cell lines in Ara-C treated mice ($p > 0.4$).

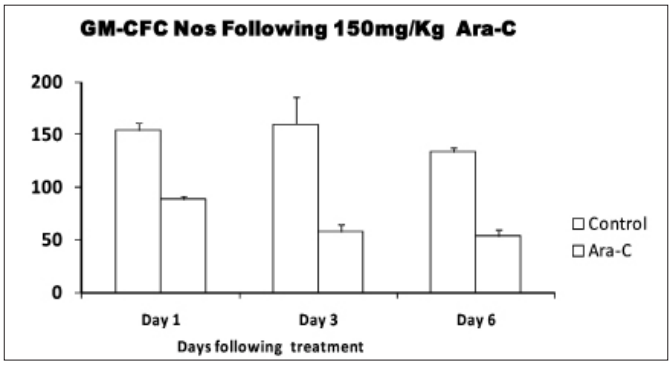
Discussion

This study has shown that Ara-C reduces the number of progenitor and stem cells at both GM-CFC and HPP-CFC levels in vivo in a murine model. Several limitations of the study need to be taken to consideration. Owing to financial constraints, we did not determine the number of nucleated cells per femur at the time of sacrificing each animal; this figure would have enabled us to determine whether cell numbers are changing as well as colony numbers. Nor did we identify the percentage of HPP-CFC in S phase, to see the proportion of these cells that would be vulnerable to Ara-C prior to treatment. Despite these shortcomings, it is possible to consider other mechanisms that might account for the differential effects that we observed (a) in the impact of Ara-C on GM-CFC and HPP-CFC cell populations and (b) in the recovery rates of these different cell populations during the days after treatment.

Possible explanations for behaviour of GM-CFC after Ara-C therapy. Ara-C mainly targets cells that are in S-phase of the cell cycle, and as 30% of GM-CFCs are in cell cycle,[12, 16-18] the majority of GM-CFC might have been directly damaged following the treatment. Most of these damaged cells can easily be replaced from the primitive haematopoietic cell population by direct recruitment. Primitive stem cells are

induced into cell cycle as well as produce progenitor cells that are mobilized from marrow to blood following cytotoxic treatment[19-21]. However, in the present study, the GM-CFC population has remained depressed partly because it has not been replaced by HPP-CFC proliferation . The period under investigation was not long enough for cell transition from the HPP-CFC compartment to the GM-CFC compartment. In absence of HPP-CFC differentiation, the GM-CFC pool would remain low or depleted with time. This could explain in part the reduction of GM-CFC numbers as the time progressed . However, one would expect a return to normal GM-CFC numbers with time as the HPP-CFC pool is recruited and replenishes the GM-CFC pool.

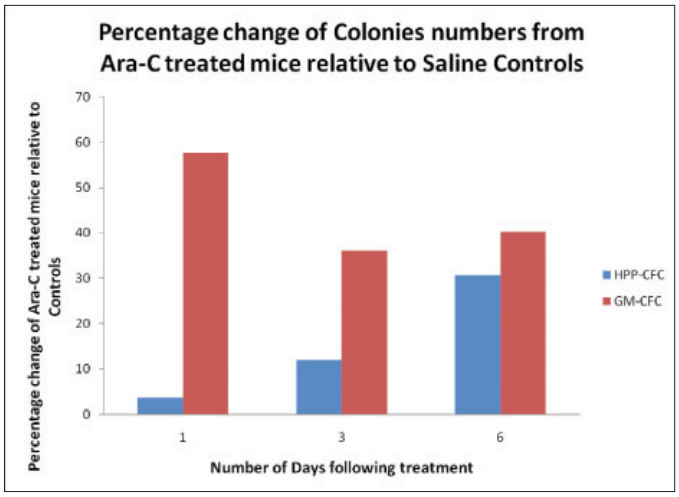
FIG. 2. Numbers of HPP-CFC per 105 nucleated cells in the bone marrow of mice injected intraperitoneally with 150mg/kg body weight of Ara-C (shaded bars) or with normal saline (open bars) on different days after treatment. HPP-CFC numbers are greatly reduced on day 1 in the Ara-C treated group, and there was a gradual increase in HPP-CFC numbers in this group over time, although the mean HPP-CFC count remained significantly lower than that in the saline group throughout the period of the study.



Effects of Ara-C on HPP-CFC

The effect of Ara-C on HPP-CFC is however more difficult to explain. One would not expect a big change in the stem cell pool following Ara-C treatment as this cell population is believed to be quiescent with less than 10% of HPP-CFC in cell cycle at any one time[12]. It is possible that a relatively high number of HPP-CFC were already in cell cycle as the mice were not specific pathogen free. However, if this were the case a relatively high proportion of GM-CFC would have also been affected as the pathogens would not only have selectively recruited HPP-CFC but also GM-CFC. More plausible explanations for the huge drop in HPP-CFC counts in Ara-C treated mice include damage to the stromal cells of bone marrow micro-environment which would lead to the destruction of the niche of HPP-CFC, HPP-CFC cell senescence; and HPP-CFC apoptosis. Stromal cell damage would destroy the niche and allow stem cells to self renew or differentiate, and hence (because now replicating) become directly vulnerable to the effects of Ara-C. In addition, the destruction of stromal cells would delay the ability of stromal cells to produce stimulatory factors that work on GM-CFC recruitment such as macrophage colony stimulating factor (M-CSF) which are expressed by accessory cells of bone marrow microenvironment for both osteoclastogenesis and macrophage maturation[22]. This in turn would reduce the recovery of the GM-CFC compartment. Since there is a feed back loop in the recruitment of both GM-CFC and HPP-CFC cell population, the inability of stromal cells to produce necessary factors crucial to keep the loop functional would result in delayed cell recovery in the GM-CFC while the HPP-CFC population would be under greater pressure to differentiate and self renew. This would therefore explain in part the increase in numbers of HPP-CFC over time and the constant numbers of GM-CFC with time following Ara-C treatment. Others have shown that Ara-C damages the bone marrow stromal cells in vivo[23].

FIG. 3. Mean numbers of of HPP-CFC and GM-CFC colonies in Ara-C treated mice expressed as percentage of mean colony numbers in controls. This figure shows that there was a statistically significant difference in colonies numbers between normal Saline Control GM-CFC and HPP-CFC colonies when compared to the Ara-C treated group .



Proliferation kinetics of cells is achieved by a series of signal transduction pathway molecules which is usually triggered by cytokines. These cytokines work as both inhibitors and stimulators of cell proliferation. Imbalance of cytokines can therefore result in disruption of the proliferation characteristics of haematopoietic cells resulting in either cell senescence, apoptosis or cell proliferation. Cytotoxic drugs can induce cell senescence[24] and also apoptosis[25]. In addition, Ara-C has been shown to accelerate recovery of in vivo inhibition of the bone marrow myeloid progenitor cells by myelosuppressive chemokines[26]. The effects of Ara-C on HPP-CFC population in the present study have shown the complexity of the Ara-C induced cell inhibition in vivo.

In conclusion, we have shown that Ara-C has a toxic effect on both GM-CFC and HPP-CFC. We suggest that the effect of Ara-C on haematopoietic cells may be due in part to concomitant damage to stromal cells. This effect could result in bone marrow damage if repeated doses of Ara-C are administered in vivo. There is need therefore for further studies to determine how stromal cells might be protected from damage during treatment with Ara-C. This will require further investigations of the bone marrow microenvironment of Ara-C treated mice – including both cellular and cytokine components – using longer term bone marrow cultures.

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medium for culturing haematopoietic cells.

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