Growth and cell cycle kinetics of AML and CLL cell line in a 3D bone marrow biomimicry under oxidative and starvation stresses

Susana Isabel Brito dos Santos

Biological Systems Engineering Laboratory, Imperial College London
Bioengineering Department, Instituto Superior Técnico

October 2013

Abstract

The implementation of the concept of personalized medicine to cancer treatments, in general, and leukaemia in particular, is an objective than has been pursued by many researchers and physicians in the last decade. This concept simply states that the best treatment for one patient is the one that takes into consideration as much patient-specific factors/characteristics as possible.

To achieve this goal which will further lead to the optimization of chemotherapy treatments, it is essential to clearly understand how leukaemia cells behave under the different conditions which are present in the human body. In this work the growth and cell cycle kinetics of leukaemia cell lines were studied in an environment that mimics the human bone marrow. Specifically, an in vitro bone marrow biomimicry system was used, which was composed of a polyurethane foam coated with collagen I, to support the growth of two leukaemic cell lines (K-562 and MEC-1) under hypoxia (5% oxygen) and at different glucose concentrations (0.9g/L, 1.3g/L and 4.3g/L).

It was concluded that the glucose availability is an important factor that conditions the cell proliferation and growth in the hypoxic environment under study. Additionally, there are also indications that hypoxia, in a 3D microenvironment, restricts the cell cycle progression with or without glucose limitations. However, some weaknesses in the used in vitro system were observed and need to be addressed in future work.

Keywords: Leukaemia, bone marrow biomimicry, haematopoietic microenvironment, oxidative stress, starvation stress.

Introduction

Leukaemia is a severe type of cancer of the blood characterized by the incapacity of blood cell progenitors to mature properly, resulting in an abnormal increase of immature white blood cells called “blasts”\(^{1,2}\). According to the American Cancer Society, in 2013, 48 610 people will be diagnosed with leukaemia in the USA, and about 23 720 people will die due to this disease\(^3\). Acute myeloid leukaemia (AML) and chronic lymphocytic leukaemia (CLL) are the most deadly types; being responsible for deaths of about 40% and 20% of leukaemia patients, respectively.

Nowadays, leukaemia treatments are based on chemotherapy, which aims to stop cancer cells from growing and reproducing. The most used cytotoxic drugs for standard leukaemia therapy almost exclusively attack the DNA replication process within malignant cells, as these cells are
selectively sensitive because of their increased proliferation. However, this approach has several limitations. Specifically, non-malignant dividing cells are also targeted, inducing a degree of toxicity that precludes treatment of some patients and forces premature termination of treatment in others. Furthermore, there is significant evidence that many tumours harbour rare quiescent clones that are resistant to this type of therapies and may be responsible for disease relapse. Since these quiescent clones are not proliferating, they are not affected by conventional treatments, thus representing an inherent mechanism of tumour resistance. Once the treatments are stopped, these cells are able to return to the cell cycle and reconstitute the tumour cell population, inducing a relapse of the disease.

Several studies have pointed out the crucial role of bone marrow (BM) niches on the evolution, i.e., proliferation, migration and adhesion of haematopoietic stem cells, as well as in cancer cells. Moreover, there are several evidences that the deregulation of the BM niche can actively influence the fate of the stem cells, being able to dictate an abnormal behaviour. The BM can be found within the bones and it is composed by an hematopoietic component (parenchyma) and a vascular component (stroma). The parenchyma includes hematopoietic stem cells and hematopoietic progenitor cells. The stroma creates a microenvironment that incorporates the 3D bone marrow niches formed by stroma cells, such as macrophages, adipocytes and fibroblasts. The stroma cells elaborate extracellular matrix proteins including fibronectin, collagen, vimentin and tenascin, which create specialised compartments with localised chemokines and cytokines resulting in the regulation of proliferation, differentiation and self-renewal of HSCs. Due to these, it is crucial to have an ex vivo culture system capable of reproducing the 3D environment of the BM and support leukaemia growth patterns similar to the ones in vivo.

On the other hand, it is been recently suggested that microenvironmental conditions can have a relevant influence in tumour progression as well as in resistance to current therapies. The occurrence of chronic oxidative stress has been demonstrated in several studies of different types of cancer, including hematopoietic malignancies such as ALL, CML and AML. The correlation between oxidative stress and malignancy is not well understood at this point. However, some studies indicated that tumour-derived ROS might induce cell survival, migration and metastasis, proliferation and drug-resistance in some types of cancer. Another cancer-related feature that has been observed, from 1930 up to date, is an increase in glycolysis pathway in almost all types of cancer, even under normoxia (Warburg effect). Several studies have been suggesting the existence of an impaired TCA cycle and blocked oxidative phosphorylation in cancer cells.

The aim of the current dissertation is to study the growth as well as the cell cycle kinetics under several environmental stresses (oxidative and metabolic stresses) through in vitro cultivation of AML and CLL cell lines in a three dimensional (3D) scaffolding system. This system has been developed to mimic the human bone marrow structure and properties. Proliferation, viability, metabolites, as well as, cell cycle kinetics analysis were performed to compare the different behaviours of the cell lines in response to different levels of stress.
Materials and Methods

Scaffold preparation

a. Foam Preparation

In order to fabricate the scaffold with the desired mechanical properties such as pore size, shape and distribution the thermally-induced phase separation procedure (TIPS) was used, with dioxane (99.8% pure, Sigma-Aldrich, Dorset, UK) as the solvent for the polymer solution of PU (5% wt). The resulting disc were previously characterized as having pore sizes from 100 to 250 μm and a porosity of roughly 90%\(^2\). The scaffolds were cut in cubes of a standard 5x5x5 mm\(^3\).

b. Collagen coating

The cubes were dipped in ethanol (70%) for 1 min followed by rinsing them in phosphate buffered saline (PBS) for 10 min. After, they were centrifuged for 10 min at 2500 rpm. Meanwhile, a calf skin-derived collagen type I (Sigma-Aldrich, Gillingham, UK) solution, with a concentration of 62.5 µg/mL, was prepared in acetic acid 0.1 M (Fisher Scientific, UK) from a stock solution. The scaffolds were dipped in the collagen type I solution prepared and centrifuged for 20 min at 2000 rpm. Then, the scaffolds were washed with PBS and centrifuged for 10 min at 1500 rpm, clearing the pores of the surface from obstructions that could hinder a deep penetration of the cell into the structure\(^10\). The cubes were subsequently distributed into individual wells in 24 well plates under sterile conditions.

c. Scaffolds sterilization

The sterilization started by dipping each one of the cubes in 2 mL of ethanol 70% for 3h, followed by two washing steps with sterilized PBS and finished by 8 minutes of UV light exposure (230 V, 50 Hz, 0.14 A, Kendro Laboratory Products UK). Finally, the wells were filled with 10% Fetal Bovine Serum-containing (FBS heat inactivated; GIBCO Invitrogen, Paisley, UK) Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen Ltd, UK) supplemented with 1% Penicillin and Streptomycin (Pen/Strep; Gibco, Invitrogen Ltd) and 0.6 g/L of glucose. The plates were incubated for 24h in a humidified environment at 37°C and 5% CO\(_2\) to assure a successful sterilization.

Cell culture

K-562 (human erythromyeloblastoid leukaemia cell line ATCC\(^{®}\), UK; CCL-243\(^{TM}\)) and MEC-1 (human chronic B leukaemia cell line ACC 497, Italy) cell lines were expanded in Iscove’s Modified Dulbecco’s Medium (IMDM; Invitrogen Ltd, UK) supplemented with 10% of FBS and 1% Pen/Strep in an incubator at 37°C and 5% CO\(_2\).

During the experiment, the cells were cultivated in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% of FBS, 1% Pen/Strep and glucose. The glucose was, afterwards, added depending on the desired conditions: 4.30g/L (control), 1.30g/L (high glucose) and 0.6g/L (low glucose). During the experiment, it was used a hypoxic incubator which was set to 5% O\(_2\), 5% CO\(_2\), 90% N\(_2\) at 37°C and with a humidified environment. The scaffolds were seeded with 0.6 millions of cells resuspended in 100μL of the respective medium and placed in the incubator for 15min and then, filled with 1.5mL of the respective medium. The cells were cultured in the referred conditions for 13 days and samples for cell analysis were taken on a daily basis.

Cell extraction

The process of cell extraction consists of aspirating the cells with a syringe, after taking the scaffold from the respective well. When the cube looked completely dried, it was discarded and the cells placed in the centrifuge tube.

Cell viability analysis

A sample of the extracted cells was stained with Erythrosin B (ATCC\(^{®}\), Manassas, VA) and counted using a standard haemocytometer, enabling the determination of the viability at that time point.

Cell proliferation analysis

The cultured scaffolds were transferred to a new 24-well tissue culture plate, where 200µL of MTS solution (Promega, CellTiter96R AQueous One Solution Cell Proliferation Assay) were added to
each well. After, each well was filled with 1 mL of the appropriated culture medium and incubated for 3h at the same conditions of the experiment. The absorbance of the coloured product was recorded at 490 nm using a 96-well plate reader (Elx 808, Ultra Microplate Reader, Bio-TEK Instruments, Inc., Bath, UK).

**DNA quantification**

The total DNA content of the cells was measured using Quant-IT PicoGreen dsDNA Reagent Kit (Invitrogen, UK). The scaffolds were placed in an eppendorf tube, in which was added 1mL of proteinase-K (Sigma-Aldrich), 50 μg/mL, in 100 mM dibasic potassium phosphate solution, pH 8.0 (K2HPO4; Sigma-Aldrich) and incubated overnight at 37°C. Proteinase-K was then inactivated at 90°C for 10 min. To further analysis, the samples were diluted 200-fold with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5; Invitrogen). 0.5 mL of Quant-it PicoGreen reagent were added to 0.5 mL of diluted sample and incubated for 2-5min. The samples were read using a spectrofluorometer (Fluoromax-4, Horiba Scientific) with the excitation wavelength set at 480 nm and the emission wavelength at 520 nm.

**Cell cycle analysis**

To perform flow cytometry, the extracted cells were diluted in 500 μL of PBS and fixed with 4,5 mL of ethanol 70% at -20°C overnight. After, the cells were centrifuged for 3 min at 300g and the pellet resuspended in 3mL of PBS. About 5 min after the resuspension, the cells were centrifuged for 5 min at 200g. The pellet was resuspended in 500 μL of a propidium iodide (PI) solution prepared, previously, by adding 10 μg/mL of PI (Sigma-Aldrich) 0.1% (v/v) Triton X-100 (Sigma-Aldrich) and 100 μg/mL DNase-free RNase A (Ribonulease A from bovine pancreas, Sigma-Aldrich) in PBS. The samples were kept in the dark, under agitation, at room temperature for at least 45min and transferred to the flow cytometer (BD Fortessa Analyser) for the cell fluorescence measurement.

**Metabolic analysis**

Samples of the exhausted media were taken in the feeding days and analysed with the Bioprofile 400 (Nova Biomedical). The values of glucose, lactate, glutamine, glutamate, NH4+, Na+ and K+ in each sample were registered.

**Statistical analysis**

The experiments where performed in duplicate (n=2 replicates per experiment) in two different occasions (N=2 separated experiments). Statistical significance of the results was evaluated using SigmaState v3.5 (Dundas Software LTD, Germany) using two-way analysis of variance (Two Way ANOVA) with a level of significance p<0.05.

**Results**

The effect of the glucose concentration in the culture medium was evaluated for two leukaemia cell lines (K-562 and MEC-1), under hypoxic environment. This study was performed in 3D system previously developed to mimic the BM microenvironment. Three different glucose concentrations were used: 0.6g/L (low glucose) that correspond to a hypoglycaemic state of the human body, 1.3g/L (high glucose) that correspond to hyperglycaemia and 4.3g/L (control glucose) that is the standard concentration in the culture media.

**Cell growth kinetics**

For the K-562 cell line (Figure 1 A), from day 2 to day 13, the control condition shows significantly higher absorbance values than the other conditions (p<0.05) and, from day 4 to day 13, the low glucose condition is the one with the smallest absorbance values. At day 2, a pronounced reduction in proliferation occurred in both high and low glucose conditions (around 50%). For the MEC-1 cell line (Figure 1 B), the absorbance values in the control condition are
significantly higher than the others (p<0.05), from day 4 to day 13. After day 7, it is also verified that the proliferation values of the low glucose condition are significantly lower than the other conditions. At day 4, it is possible to notice a severe reduction in proliferation, in all conditions. This reduction has particular relevance for the low and high glucose conditions (around 50% and 20%, respectively).

For both cell lines (Figure 2), is just possible to statically distinguish the control conditions as the one with higher cell number (at days 3, 4, 6, 7, 9 and 13).

Generally, the DNA quantification technique showed that the cell number of K-562 is higher than the MEC-1, comparing the same condition at the same time point.

Cell cycle analysis

The cell cycle analysis (Figure 3) showed that for both the cell lines under study, the majority of the cells are in G1 phase for all the conditions (between 45% and 65% of the K-562 cell population and between 49% and 67% of the MEC-1 cell population). Also the percentage of cells in G2/M remained approximately constant during the time of the experiment and did not differ considerably between conditions (around 15% for K-562 and 10% for MEC-1). In K-562, for all the
conditions, it is possible to notice that the percentage of cell in G\textsubscript{1} evolves through a sequence of a valley and a peak, having a minimum at day 3 and a maximum at day 10. The percentage of cells in the S phase follows an inverted trend, which is similar for all the conditions. In MEC-1, for the high glucose condition, the percentage of cells in G\textsubscript{1} has a maximum at day 6 (with a correspondent minimum in the percentage of cells in S). For control glucose, there was an increase of the percentage of cells in G\textsubscript{1} until day 6, followed by a stabilization of this percentage. For low glucose, a slight decrease of the percentage of cells in G\textsubscript{1} was registered over time.

Metabolic analysis (data not shown)

For both cell lines, the measurement of the glucose concentration in the exhausted medium was not possible in high and low glucose conditions because it was below the limit of detection of the instrument (1.1 mmol/L).

For the K-562 cell line, the lactate production for the high glucose condition is around 2 times higher than the lactate production for the low glucose condition and it remains approximately constant during the time of the experiment. In the control glucose condition, it is noticed a slight increase of the lactate concentration throughout.
In this condition, the concentration of lactate registered is around 5 times higher than in the low glucose condition and around 1.5 times higher than in the high glucose condition. It is also verified that the glucose concentration in the control condition has a severe reduction from day 0 to day 2, being consumed approximately 12 mmol/L during this time. After that, it decreases over time, reaching values close to the limit of detection of the instrument at day 12. Regarding the amino acids glutamine and glutamate for the K-562 cell line, it is possible to verify the consumption of glutamine (approximately 1.5 mmol/L every two days) during the time of the experiment. Differences in glutamine consumption between conditions are not verified. Low levels of glutamate production (0.2-0.3mmol/L every two days) were also registered during the experiment for all conditions.

For the MEC-1 cell line, the glucose concentration in the control condition suffers a severe reduction from day 0 to day 2, being consumed approximately 9 mmol/L. After that, the concentration of glucose in the exhausted medium seems to be stable over time. The lactate production for the high glucose condition is also around 2 times higher than the lactate production for the low glucose condition and it remains approximately constant during the time of the experiment. In the control condition, the lactate production is similar to the one in the high glucose condition until day 6, but after that it increases, reaching values almost 2 times higher. The consumption of glutamine in MEC-1 is smaller than in K-562 (around 1 mmol/L every two days) and constant for the length of the experiment. The glutamate production is very small or inexistent. It was not possible to distinguish between conditions in the measurements of the concentrations of these amino acids.

**Discussion**

Both the leukaemia models were successfully cultivated in the 3D system for 13 days under the studied conditions. It was observed that both the cell lines proliferate during this period. As would be expected, the proliferation is lower under severe starvation conditions (low glucose) in both the cell lines. For both cell lines, the control glucose condition is the one that shows higher proliferation. This fact is not surprising since, according to the selling company, these cell lines were developed and optimized to be cultured at this glucose level. Together, these data indicates that glucose availability in the media is a factor that critically conditions cellular proliferation, at least under hypoxia.

A severe decrease of proliferation in both cell lines was also observed, for K-562 at day 2 and for MEC-1 at day 4, which is probably, caused by the introduction of the stress factors (hypoxia and glucose starvation). In K-562, this decrease is equally noticed in low and high glucose conditions (approximately 50%) while in the control glucose condition it is almost inexistent (6%). In MEC-1, the proliferation is reduced in 50% in the low glucose condition and in 20% for high and control glucose conditions. These differences both in the duration of the lag phase as in the intensity of the decrease can be justified by the different cancer metabolism. While the K-562 has a fast metabolism (doubling time of 12h) that will easily be affected by changes in the environment, the MEC-1 has a slower metabolism (doubling time of 40h) that will take more time to respond to environmental changes.
The cell cycle analysis indicated that the majority of the cell population was stacked at G0/G1 in both cell lines, most probably, as consequence of the induced stress in all the studied conditions. For K-562, the percentage of cells in G0/G1 and S phases follows a trend of peaks and valleys that suggest that the cells are exposed to cycles of adaptation and steady-state followed by a new adaptation period. In adaptation periods, the percentage of cells in S phase is reduced in the beginning and increases with time, reaching a peak that correspond to a steady-state. The opposite happens with the percentage of cells in G0/G1 phase, which is higher in the beginning of the adaptation phase and decreases when the culture is evolving towards the steady-state. For MEC-1, the observed trend is slightly different. In that case, for the high glucose condition the percentage of cells in G0/G1 increases until day 6, in which it has a maximum, and starts to decrease during the following days, while the percentage of cells in S followed an inverted trend. For the low glucose condition, the percentage of cells in each one of the cell cycle phases remained approximately constant. Finally, in the control glucose condition, the percentage of cells in G0/G1 increased until day 6 and remained approximately constant after that. Consequently, the percentage of cells in S followed an inverted trend. The percentage of cells in G2/M is similar between the conditions and it does not show considerable variations over time.

For both cell lines, it was calculated the ration between the percentage of cells in G0/G1 and the percentage of cells in S (data not shown) in order to verify if this ratio is influenced by the glucose availability in the medium. It was verified that for K-562, this ratio is higher for the low glucose condition in most days. This result was expected because if the cell does not have the required amount of glucose available to fulfil its nutritional needs, it will not be able to progress in the cell cycle, being staked in G1 or adopting a quiescent state (G0). Surprisingly, the opposite was observed for MEC-1. The ratio between the percentage of cells in G1 and the percentage of cells in S was higher for the control condition in most days. It was not possible to find any data in the literature that supported this observation.

In spite of the present conclusions, it is not possible to ignore the variability observed, which is most probably, due to the culture system and extraction method, and also the percentage of the cells used for the cell cycle analysis. During the time of the experiment, an increase in the number of debris among the total population analysed by flow cytometry was evident. This may lead to an unrepresentative analysis of the cells’ behaviour and to biased conclusions if the live cell population is too small.

Until now, it was not possible to find a reliable method that enables the extraction of the large majority of the cultured cells from the scaffolds without inducing damages that would compromise the subsequent conclusions.

The metabolic data suggests that the glucose consumed by cells in high and low glucose conditions are mainly being used to cell maintenance while, in control condition, there were enough glucose available to use it in the cell growth. This suggestion can be supported by the large difference existent in the cell number and proliferation of the high and low glucose conditions when compared with the control condition. It can be speculated that the small proliferation observed in these two conditions is mainly supported by the...
glutamine metabolism (particularly in the K-562 cell line in which high glutamine consumption is observed). This idea can be supported by Lodi et al. (2011), in which it was demonstrated that in hypoxic conditions (1% O₂) the glycolysis is enhanced, but the concentrations of metabolites within the TCA cycle are, except for citrate, not significantly altered in the K-562 cell line. Tanking these results into account and the consumption of glutamine observed here, it can be speculated that glutamine is being transformed into α-ketoglutarate, which then feeds the TCA cycle, allowing for the production of the other intermediates. This is a common observation among cancer cells, in which the glutamine represents a fundamental substrate in cases of glucose starvation (DeBerardinis et al. 2010).

Finally, it would be interesting to perform 2D experiments that would allow the distinction between the environmental stress and the effect of the 3D microenvironment on the cells. Additionally, it would also be interesting to perform an experiment with an intermediate level of glucose -for example around 2g/L- in order to investigate the effect of mild glucose stress on the cellular evolution without partial starvation exposure. Furthermore, future experiments should be conducted under normoxic conditions, i.e., 20% oxygen, in order to isolate the effect of the oxygen concentration in the behaviour of the cells.

Conclusion

In summary, this work showed that it is possible to culture two cell lines (AML and CLL) in the 3D polyurethane scaffold-system previously developed, under oxygen and glucose restrictions, for 13 days. Although the several advantages associated to the use of this 3D system, due its BM-mimicking structure, there are also some technical limitations that raise various issues in the conclusions obtained. These technical limitations may explain the absence in the literature of alternative 3D systems able to be used as platforms to perform leukaemic studies. Despite the limitations, it was proven that glucose availability is a crucial factor for cell proliferation and growth under hypoxic environment. Another important hint that arises from this work is that hypoxia, in a 3D microenvironment, restricts the cell cycle progression, with or without glucose limitations. However, this idea has to be confirmed in further studies.

In conclusion, this work contributed to the understanding of the effect of the oxidative and starvation stresses in leukaemia simultaneously with the elucidation of the limitations of the system used.

References


