Medical Bio Trial Use Proposal

1. Proposal number : 2008B1960

2. Title of experiment: Valence states of hemin-iron in cultured brain cells: implications for haemorrhagic stroke

3. Name and affiliation of the project leader: Dr Marian Cholewa
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4. Beamline used: BL37XU

5. Research purpose and background

Stroke is the third leading cause of death in Australia and one of the main causes of disability. Around 20% of strokes involve the rupture of cerebral blood vessels, and these 'haemorrhagic strokes' have a higher degree of lethality than other forms of stroke. As the haemoglobin from red blood cells oxidises, the hemin (oxidised heme) and globin moieties separate from each other. It is widely believed that the release of iron from hemin is one of the main causes of the brain damage that follows a haemorrhagic stroke. The popular view in the literature is that the hemin is subsequently broken down by heme oxygenase-1, thereby releasing iron that can catalyse the generation of cytotoxic hydroxyl radicals, particularly when in the presence of superoxide and hydrogen peroxide, which are normal byproducts of cellular metabolism (particularly in mitochondria).

Experiments in the laboratory of Robinson and Bishop have cast doubt on this popular view. We have confirmed that cultures of primary brain cells (astrocytes or neurones) do readily take up hemin, and that it is very toxic to these cells. However, we have shown that the cells do not metabolise much of the hemin that they take up. Therefore, it appears that the toxicity of hemin could be due to hemin itself, rather than to its breakdown products (iron and biliverdin/bilirubin). We now have evidence that hemin is able to directly interact with hydrogen peroxide, and we hypothesis that the iron remains bound to the protoporphyrin ring within the hemin while it participates in the Haber-Weiss/Fenton reaction to produce toxic hydroxyl radicals. If this hypothesis is correct, then the iron moiety in hemin must cycle between Fe^{2+} and Fe^{3+}, rather than remaining oxidised as Fe^{3+} as had been previously supposed. The present project will examine whether the iron in hemin does cycle between both valence states when taken up into brain cells.

In this experiment, astrocytes will be isolated from neonatal rat brains and then culture in 24-well plates for 14 days until semi-confluent. Pathophysiological concentrations of hemin will then be added to the culture medium, and the cells will be incubated for a period ranging from 15 minutes to 24 h. The cultures will then be washed 2 times with phosphate-buffered saline before being lifted from the culture wells using trypsin. Cells will then be washed in 200mM ammonium acetate and dropped onto a Kapton foil. Cells will then be fixed in isopentane cooled in liquid nitrogen and freeze dried. Experiments by other investigators have shown that this fixation regime greatly limits the rate of oxidation of the tissue sample, and renders it suitable for examination with synchrotron hard X-irradiation. The samples will be examined on the BL37XU beamline at the SPring-8 synchrotron, and the distribution of iron within the cells examined. The BL37XU beamline offers a spatial resolution below 1 μm which will be sufficient for this project. We should be able to conduct elemental characterization by

employing a synchrotron radiation-induced X-ray emission (SRIXE) technique. Chemical characterization will be conducted by using the X-ray absorption near edge spectroscopy (XANES) technique. Data on the relative frequency of the two valences of iron within different intracellular compartments, may enable us to determine which organelles are most vulnerable to the toxicity of hemin. Such an investigation is not possible with conventional microscopy. The ratio of Fe^{2+}/Fe^{3+} within the cells will be compared to that within samples of pure hemin to demonstrate whether a significant proportion of the intracellular hemin-iron has been reduced to Fe^{2+}. It may also prove possible to determine whether the reduction of hemin-iron occurs at the cell membrane, in association with particular organelles (eg. mitochondria), or uniformly throughout the cytoplasm. It would not be technically possible to compare the intracellular distribution of Fe^{2+} and Fe^{3+} without a synchrotron X-ray beamline, and no such beamline currently exists at the Australian synchrotron. The data obtained in this study should provide important insights into the basis of hemin toxicity, and may help to point towards new avenues for the therapeutic treatment of haemorrhagic stroke.

6. Experimental/analytical method: SRIXE (Synchrotron Radiation Induced X-ray Emission)
XANES (X-ray Absorption Near Edge Spectroscopy)

7. Research results

For the experiments at the BL37XU beamline at SPRING8 we prepared three groups of samples. The first sample group consisted of astrocytes that were grown in their normal culture medium. The second sample group consisted of astrocytes that were incubated with hemin for 24 h. The final sample group consisted of astrocytes that were incubated with free iron from ferric ammonium citrate (FAC) for 24 h. For each sample group we targeted at least 10 individual cells and obtained elemental maps at one micron resolution for iron, copper, zinc, calcium, potassium and phosphorous for each cell. We also obtained elemental spectra from 2 subcellular locations (nucleus and cytoplasm) and the background outside the cell, in order to quantify these elements. For the sample groups that were incubated with hemin or FAC, we conducted XANES analyses of iron in order to determine the valence state of iron.

Preliminary results from these analyses show that the iron content of cells incubated with hemin or FAC contained high levels of iron. The level of iron in these cells were found to be about 10 times higher than in control (untreated) cells. The elemental maps suggest that the hemin accumulated by astrocytes is diffusely located throughout the cell body. By contrast, the iron accumulated from FAC was highly localized to a perinuclear location. Taken together, these results suggest that hemin may be able to exert its toxic effects throughout the cell body while free iron is localized to a specific area within the cell, perhaps to limit its toxic effects. Preliminary XANES analyses show that both Fe^{2+} and Fe^{3+} are present in cells incubated with hemin, indicating that hemin is reduced to heme. These data support the hypothesis that hemin is toxic to cells by producing radicals through cycling between valence states. In contrast, iron in cells treated with FAC was found to be wholly Fe^{3+}, indicating that the iron is unlikely to undergo redox cycling, and may be stored within iron storage proteins such as ferritin.

8. Current and future issues/challenges

During the experiment we have identified two groups of issues/challenges:

A. Sample environment

Samples have been prepared on a large plastic (acrylic) frames with holes covered with a thin (7.5 μm thick) Kapton foil. A small (5 μL) of liquid medium with about 100 cell was deposited on a Kapton foil and freeze-dried. Prior to experiment at the BL37XU beamline, cells were identified and their position recorded with the use of an off-line microscope. We have identified two issues:

1. **Offset in cell position:** there is an about 50 x 80 μm offset between the stage coordinates on the off-line microscope and the on-line stage at the beamline.
2. **Distance of cell to the detector:** with the present beamline geometry (10 degree tilt) the detector could only be positioned at a large distance (approximately. 30 mm) from the cell during data collection. This limited the solid angle of the detection system and reduced data collection rate.

B. Data collection and analysis

We have identified two issues:

1. **Number of elemental maps:** Data could only be collected from a maximum of 6 pre-selected energy windows, which constrained the range of elemental maps that could be collected during the experiment. If some other elements might become of interest later on it would be necessary to repeat the experiment. Data collection in the event-by-event mode (full spectra collected for each pixel) will solve this problem in the future.
2. **Peaks overlap and background:** at present data collection does not allow the user to obtain precise quantitative information about elemental concentration. When selecting an energy window is not possible to eliminate overlaps of peaks from different elements, pile-up peaks and subtract the background.

9. References

Not applicable.

10. Status of publication and patent

During the time allocated for our experiment we have collected very useful SRIXE and XANES data. And we are planning to publish these results in a journal with a high impact factor. We have not yet decided which journal this will be, and a decision will be made until after data analysis has been completed.

11. Keywords and annotations

Keywords: astrocytes, rat brain, elemental analysis, oxidation state of Fe

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