The Ultrasoft X-ray Microbeam : a sub-cellular probe of radiation response.

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A new microbeam facility has been developed at the Gray Laboratory in collaboration with the King's College London. This microbeam uses diffraction optic techniques to focus ultrasoft carbon K X-rays (278 eV) to a spot of sub-micron size which can be used to irradiate single cells *in vitro.* Characteristic carbon X-rays deposit their energy through the photoelectric effect, to produce about 14 ionisations in a 7 nm range in biological tissue. Focusing the X-rays in a very fine beam will allows us to produce very localised clusters of energy deposition inside the biological samples. The extent to which the ionisations are clustered can be precisely controlled, and reduced to the size of critical sub-nuclear targets such as higher-order DNA structures. The radiobiological importance of clusters of ionisations has been highlighted both by Monte Carlo track-structures simulations (1, 2) and by experimental studies (3, 4). Damage arising from clustered ionisations is considered particularly critical for the cell, as repair is expected to occur less efficiently with increasing the damage complexity. The ultrasoft X-ray microbeam represents a unique tool for studying the relationship between radiobiological effect and the size of the clusters of ionisations produced. It is possible to vary independently both the flux of photons and their focused spatial distribution. In some studies we have substituted a laser plasma microfocus X-ray source (308 eV, single 7 ps pulse) to mimic certain features of high LET tracks. In addition, the fine spatial resolution achieved with the X-ray microbeam can be used to assess how radiosensitivity is distributed across the cell. Contrasting data have been reported regarding the sensitivity of different regions of the cell nucleus. Using α-particles, Raju (5) and Lloyd (6) found that the most radiosensitive sites are located in the middle of the cell nucleus while Cole (7) indicated the DNA close to the nucleus membrane as main target. The identification of the major radiosensitive sites inside the cells represents an important step in analysing data and in designing new experimental approaches. Should the chromatin be considered the major radiosensitive material, cells will have to be considered systems in which the spatial distribution of such material changes dramatically during the cell cycles. The ultrasoft X-ray microprobe offers also the possibility to carry out "single cell experiments" in which only pre-selected cells can be irradiated and the radiation effect assessed following the fate of each irradiated cell and its unirradiated neighbours. This will provide a new and very accurate approach to investigate the bystander effect measuring critical parameters such as the range of intra-cellular interactions.

Characteristic carbon X-rays are produced by bombarding a graphite target with electrons accelerated up to 30 keV. The electrons are produced by a heated tungsten filament and subsequently focused onto the graphite target by a water cooled electromagnetic lens to produce a "point" X-ray source (less than 4 μm diameter). The high energy photons (bremsstrahlung), also produced by the electron bombardment, are eliminated using shallow angle reflection on a silica mirror. Measurements indicate that a nearly monochromatic X-ray beam (> 95 % beam purity) is achieved for a 2 degree reflection angle while the carbon K component is reduced by less than 30 %.

The main X-ray focusing element is a zone plate which is a circular diffraction grating, typically 200 µm in diameter, with a radially increasing line density (8). A specific set of masks (i.e., a 30 µm diameter apodized spot and a 12 µm pinhole) has to be accurately aligned with the zone plate in order to avoid background and high order focused radiation from reaching the samples. Accurate measurements performed by scanning a knife edge mask across the pinhole, indicate that the emerging X-ray beam is focused into a less than 250 nm radius spot. The present X-ray flux (about $1.5\ 10^3$ carbon K X-ray per second) corresponds to a dose rate of about 0.15 Gy / s averaged over a typical mammalian cell. This low dose rate together with the high stability of the X-ray source (less than 5 % fluctuation on the dose rate over a period of 3 second) and the possibility to measure radiation effect on a single cell basis makes the microprobe particularly suitable for low dose studies. Computerised image analysis and micropositioning techniques are also employed to perform fast and accurate experiments. The system is similar to that used for the charged particle microbeam (9). It is based on a three-axis micropositioning stage (precision of 250 nm / motor step) and an epi-fluorescent UV microscope with intensified CCD camera coupled to a fast PC. Biological targets are stained with fluorescent dye (e.g, Hoechst 33258 for the cell nucleus) and viewed under UV illumination (UV exposure of about 30 ms per cell) using a ×10 or ×40 objective. Using in-house developed software, about 100 samples can be correctly located and aligned with the probe in less than 5 minutes during a completely automated experiment.

A single cell survival assay has been established in preliminary experiments using a V79 cell line. The cells are seeded on a 0.9 µm mylar film (65 % transmission for carbon K X-rays) and, once attached (about 4 hours), their nuclei are stained with a fluorescent dye (1 µM of Hoechst 33258 for 1 h). The cell nuclei are then located by scanning the cell dish under UV illumination and recording their positions. These co-ordinates are subsequently used to position the sample over the probe. The desired dose is then delivered by exposing the samples to the X-ray beam for a pre-determined period (based on cell morphology and dose rate). After irradiation, the cells are incubated for 3 days, then individually revisited (using again the recorded co-ordinates) and the presence of surviving colonies (≥ 50 cells) assessed. Measurements performed in the dose range (0.25 Gy) delineate a linear quadratic survival curve in good agreement with data obtained using conventional soft X-ray irradiation techniques. In particular the low survival value (1.2 ± 1.1 %) measured following 2.5 Gy indicates a correct dose delivery while the small effect detected after 80 mGy underlines the possibility to perform precise low dose experiments. The microprobe data do not show a hypersensitive response at low dose that has been previously detected using the charged particle microbeam (3.2 MeV and 1.0 MeV protons) and 240 kVp X-rays (10). However, this is not in contradiction with the induced radioresistance hypothesis considering the high biological effectiveness of the carbon K X-rays (the hypersensitivity response has been found to reduce by increasing the LET (11, 12) and the localised irradiation technique. The complex lesions produced by the carbon K photons and the localisation of the dose deposited could minimise the effect of the repair mechanisms triggered at low dose.

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