Radiobiological Studies Using Synchrotron-produced Ultrasoft X-rays

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Ultrasoft X-rays have been extensively used to explore radiobiological mechanisms surrounding cell killing. These studies for the most part have been linked to a small number of X-ray energies. Recently, this field of study has been broadened by the availability of synchrotron-produced ultrasoft X-rays which can be produced at any desired energy. We have taken advantage of the University of Wisconsin Synchrotron to reexamine two fundamental radiobiological questions: Does RBE vary with different ultrasoft X-ray energies? Does the fraction of the nuclear volume exposed to equal total X-ray energy modify cell cytotoxicity?

The first study focuses on the survival of Chinese hamster V79 and mouse C3H10T1/2 cells irradiated with synchrotron-produced 273 eV and 860 eV ultrasoft X-rays. These two energies, which are available by multilayer monochromatization of the synchrotron output spectrum, exhibit equal attenuation within living cells. Such an isoattenuating energy pair allows the direct examination of how biological effectiveness varies with the energy of the ultrasoft X-rays. In comparing survival results, we find similar biological effectiveness of these two energies for both the C3H10T1/2 and the V79 cells. These results are consistent with previous findings of increasing RBE with decreasing ultrasoft X-ray energies. In addition, after correcting for mean nuclear dose based on measurements of cell thickness obtained with confocal microscopy, we find no significant differences in survival between the two ultrasoft X-ray energies and 250 kVp X-rays. These results suggest that RBE does not increase with decreasing energy of ultrasoft X-ray between 860 eV and 273 eV.

In a second study we introduced an method which allows partial-volume irradiation of live cells using synchrotron-produced ultrasoft X-rays and micro-fabricated irradiation masks. The masks were made by X-ray lithography at the University of Wisconsin Synchrotron Radiation Center, and they consist of 1.85-μm-wide stripes of gold 1.35 μm apart plated onto thin silicon nitrate membranes. When placed adjacent to mylar on which live cells are plated, these masks allow cells to be irradiated in a striped pattern with dimensions much smaller than the cell nuclei. Using 1340 eV synchrotron-produced X-rays, we compare the survival of cells subjected to uniform irradiation and cells subjected to partial-volume irradiation. Our results show that, at equal mean dose to the nucleus (i.e. equal total energies deposited), survival is not statistically different for the two treatments over a wide range of doses. Thus, imparting equal energies to...
smaller intranuclear volumes does not appear to modulate cell killing.

**INTRODUCTION**

Ultrasoft X-rays (>5 keV) have been used over the last half century to study the mechanisms by which radiation interacts with cells to cause biological changes. Ultrasoft X-rays physically interact with biological molecules exclusively through low energy photo and auger electrons. Interestingly, these electrons deposit their energy in a very small area of nanometer dimensions. This target area is in the same range of cellular molecules including chromosomal and DNA diameters. In contrast to higher energy X-ray and gamma ray photons, with their complex tracts of ionizations, ultrasoft X-rays with their well defined secondary particle tracts can be used directly to relate properties of radiation energy deposition to biological endpoints. In addition to this utility, because of their relatively low energy, they can be collimated at micron resolution allowing them to be used as subcellular ionizing probes.

In this review we will discuss two central radiobiological question that can be addressed by the use of ultrasoft X-rays. This will be used to demonstrate the great utility of using synchrotron produced monochromatic ultrasoft X-rays in addressing radiobiological questions. The first question to be reexamined with this improved source of ultra soft X-rays addresses the relative biological effectiveness of track-end electrons in comparison to the entire track electrons produced by hard X-rays and gamma rays. The second question examines the outcomes of partial versus total nuclear irradiation on cell cytotoxicity.

**METHODS**

In this Section we will only summarize the methods used. For full details the reader is referred to the original publications.

*Synchrotron beam line*

Previously, most mammalian cell-ultrasoft X-ray studies were confined to using a very limited set of X-ray energies which were available from conventional X-ray sources. A synchrotron radiation source presents the possibility of a continuous spectrum of ultra-soft X-rays at high intensities. We used the University of Wisconsin electron storage ring (synchrotron) Aladdin. Aladdin operates at 0.8–1.0 GeV and has a 2.0833-m radius of curvature. In order to use this radiation source to produce the needed ultrasoft X-ray energies at high intensity, we designed and built a double multilayer mirror monochromator. This monochromator yields a usable photon range of 250 eV-2400 eV. The resolution was ΔE/E = 0.04 and the attenuation coefficient of any selected monochromatic beam within this output range can be accurately determined within 3%.

The next challenge in designing this beam line was to pass the beam from the monochromator which is operated at high vacuum to cells being maintained in an aqueous environment. To accomplish this, cells are grown in 25 mm steel dishes with 3 μm mylar film bottoms so that the
bottom surface of the dishes face the scanning beam (1.6 mm × 25 mm). In between the monochromator and the dish is a filter that both serves to eliminate long wavelength radiation that is specularly reflected from the mirror surface and also acts as a vacuum seal to separate the monochromator from the biology chamber (details are given in Ref. 2, 3 and 4).

Dosimetry

Dosimetry is based on energy fluence at the cell surface, the shape of the cell, and the rate of cellular attenuation of the radiation. The energy fluence at the cell surface is measured by photodiode with a 3.63 eV per electron hole pair theoretical response over most of the ultrasoft X-ray region.

The shape and size of the cells (V79 and 10T1/2) were measured under a similar environment as they were irradiated under. The important parameter of thickness was measured by scanning laser confocal microscopy using the reflected light mode. This is more accurate than fluorescence imaging in that fluorescence focal spot “blooming” is avoided (see Ref. 3 for dosimetry and microscopy details).

Irradiation masks for partial cell exposure

In order to be able to irradiate a fixed percentage of each nucleus of a large population of cells, an irradiation mask (made by X-rays lithography) is placed next to the mylar dish bottom on the beam entry side. The mask is made of opaque “stripes”. The striped pattern resembles a diffraction grating with gold lines of approximately 1 μm in thickness. The irradiation energy used was 1340 eV. It was chosen for good cellular penetration and almost complete absorption

Fig. 1. Schematic for the irradiation of cells. Cells are plated to a confluent monolayer on mylar previously affixed with epoxy to the cell dishes and pretreated with growth serum to enhance cell attachment. Before irradiation, the cell dishes are in turn filled with cell growth media and sealed on the non-mylar side with sterilized parafilm.
by 1μm of gold (99.5% absorption). Only 10T1/2 were used in these experiments due to their reduced thickness (3–5 μm) (Fig. 1).

RESULTS

Irradiation with an isoattenuation pair of ultrasoft X-rays

In order to determine if previously reported results of differing cell survival with different ultrasoft X-ray energies (more killing with lower energies) was due to the effects of energy or unaccounted for attenuation differences, we irradiated both 10T1/2 and V-79 cells with ultrasoft X-ray beams of differing energies but identical attenuations. This was achieved by taking advantage of the various absorption “edges” associated with the mass fraction average of the nuclear atomic composition. The energies chosen were 273 eV and 860 eV. Both have an attenuation of 0.55 mm⁻¹ (3).

Since the cellular attenuation of these two energies are equal, surface dose is an appropriate parameter to compare to cell survival in order to determine the effects of these differing ultrasoft X-ray energies of cell survival. As shown in Fig. 3 no differences in survival between these two energy photons are found for either the thick V-79 cells or the thin 10T1/2 cells.

With appropriate caution we also attempted to convert surface dose to mean nuclear dose using thickness measurements made using confocal microscopy in the light scatter mode. This gave a correction value for 10T1/2 of 0.39 and for V79 of 0.13 based on an attenuation coefficient of 0.55 mm⁻¹. If one assumes these dose extrapolations to be reasonably accurate then the

![Fig. 2. A plot of X-ray attenuation through 1 μm of cell nucleus. Attenuation values are calculated as mass fraction averages of the nuclear atomic composition as defined by ICRU. The energies 0.273 V and 0.860 keV have equal attenuation values as indicated by the dotted line.](image)
Fig. 3. 10T1/2 survival fits at 0.273 keV and 0.860 keV compared to 250 kVp X-rays. Mean dose for the ultrasoft energies was calculated using the attenuation factor and the cell thickness measurements.

survival of cells at both these energies can be compared to each other and to 250 kVp X-rays. When this is done no significant RBE is found between these energies of ultrasoft X-rays and 250 kVp X-rays (Fig. 3).

Cell killing by whole versus partial nuclear irradiation

10T1/2 cells grown to confluence to maximize cell-thickness uniformity of between 3 and 5 μm were irradiated growing on 8 μm mylar. Pairs of masked and unmasked cultures were irradiated to equal nuclear imparted energies (Fig. 4). The resulting survival curves were statistically indistinguishable for cells uniformly on partial irradiated with a series of exposures of equal total nuclear dose (Fig. 5).
**Fig. 4.** A schematic illustrating the differences in energy event separation for partial volume irradiation (PI) vs. uniform irradiation (UI). The geometry of the irradiation masks creates 1.35 \( \mu m \) stripes of localized photoelectric interactions separated by 1.85 \( \mu m \). In order to achieve equal mean dose between treatments, the partial irradiation treatment deposits 2.38 times the energy into the smaller volume.

**Fig. 5.** Least-squares fits to the pre-tailing portions of the survival curves. \( \beta \) values are constrained to \( \beta = 0 \), consistent with the linear dose response. Fitted \( \alpha \) values for the partial vs. whole cell irradiation treatments were different by only 6%. Entrance dose is represented by the incident X-ray energy averaged over the whole cell surface, as explained earlier.
DISCUSSION

Two fundamental radiobiological questions were addressed in this review through the use of monochromated ultrasoft X-rays. The first question addressed the effect of isoattenuating ultrasoft X-ray energies on cell survival. Initial work by Goodhead and others using V-79 cells suggested that at equal calculated mean nuclear dose low energy ultrasoft X-ray were more effective than those of higher energy\textsuperscript{1}. Goodhead’s findings with V-79 cells were confirmed by the Los Alamos group\textsuperscript{5}.

However, they could not generalize this result for the thinner cell line 10T1/2\textsuperscript{6}. Experiments with human diploid fibroblasts which are also a thin cell strain also could not confirm findings with V-79 cells\textsuperscript{7}. This conundrum in which thick cells show an energy dependent level of killing which is not seen for thin cells may be due to the inability of method used to reliably extrapolate surface dose to mean dose. The thicker the cell the larger this variance would be possibly leading to errors in calculating dose-survival statistics. In order to remove this possible confounding factor in extrapolating mean cell dose from surface dose, we compared cell survival following irradiation with ultrasoft X-rays of two differing energies but the same attenuation coefficient through cellular material. We reported equal cell survival at 273 eV and 860 eV. For both thin (10T1/2) and thick (V-79) cells. Our data suggest that when known surface doses of isoattenuating energies are used to kill cells, no effect of photoenergy on cell killing is observed\textsuperscript{3}.

We also, with caution, attempted to estimate mean nuclear dose from these ultrasoft energy surface doses. Our estimates of cell thickness, the key to dose calculation for isoattenuating X-ray energy pairs, was determined in live cells using the best available confocal microscopy methodology using light scatter imaging. Our estimates of cell survival versus mean nuclear dose was compared for cells irradiated with ultrasoft and 250 kVp X-rays. No significant RBE was measured\textsuperscript{3}. Together these data suggest that the end-track electrons of conventional hard X-ray or $\gamma$-ray is no more effective in cell killing than electrons over the entire track.

Partial cell irradiation

The geometric location of critical targets within a cell nucleus for cytotoxicity is unknown. In order to begin to acquire data to address this question, we compared the effects of irradiation given to the entire nucleus or to only approximately half of the nucleus using synchrotron-produced ultrasoft X-ray and a gold striped mask. We hypothesized that if it was necessary to inactivate multiple specific discrete targets located throughout the nucleus, we might see less killing for equal total dose in the partially shielded cells. In contrast, if killing cells required greater than one damaging event in close proximity such as double-strand breaks, then more killing might be observed in the partially irradiated cells. Our results of equal cell killing in both irradiation conditions do not support either hypothesis.

In summary, we have used the University of Wisconsin synchrotron to address two fundamental radiobiological questions. Our approach and results clearly demonstrate the great potential for synchrotron-produced ultrasoft X-rays in the investigation of radiobiological mechanistic questions.
REFERENCES

1. Goodhead, D. T. (1994) Soft X-ray radiobiology and synchrotron radiation. In: Synchrotron Radiation in the Biosciences, Eds., B. Chance, J. Deisenhofer, S. Ebashi, D. T. Goodhead, J. R. Helliwell, H.E. Huxley, T. Iizuka, J. Kirz, T. Mitsui, E. Rubenstein, N. Sakabe, G. Schmahl, H. B. Stuhrmann, K. Wutrich and G. Zaccai, pp. 683–705, Oxford University Press, New York.

2. Mackay, J. F., Pearson, D. W., Nelms, B. E., Deluca, P. M., Jr. and Gould, M. N. (1998) A double mirror W/C multilayer monochromator for radiation biology applications. Med. Phys. 25:773–779.

3. Hill, C. K., Nelms, B. E., MacKay, J. F., Pearson, D. W., Kennan, W. S., Mackie, T. R., DeLuca, P. M., Jr., Lindstrom, M. J. and Gould, M. N. (1998) Synchrotron-produced ultrasoft X-rays: equivalent cell survival at the isoattenuating energies 273 eV and 860 eV. Radiat. Res. 150: 513–520.

4. Nelms, B. E., Mackie, T. R., MacKay, J. F., Hill, C. K., DeLuca, P. M., Jr., Lindstrom, M. J., Deasy, J. and Gould, M. N. (1998) A comparison of cytotoxicity after whole-or partial-cell irradiation with synchrotron-produced ultrasoft X-rays. Radiat. Res. 150: 521–527.

5. Raju, M. R., Carpenter, S. G., Chmielewski, J. J., Schillaci, M. E., Wilder, M. E., Freyer, J. P., Johnson, N. F., Schor, P. L., Sebring, R. J. and Goodhead, D. T. (1987) Radiobiology of ultrasoft X-rays. I. Cultured hamster cells (V79). Radiat. Res. 110:396–412.

6. Schillaci, M. E., Carpenter, S., Raju, M. R., Sebring, R. J., Wilder, M. E. and Goodhead, D. T. (1989) Radiobiology of ultrasoft X-rays. II. Cultured C3H mouse cells (10T1/2). Radiat. Res. 118:83–92.

7. Cornforth, M. N., Schillaci, M. E., Goodhead, D. T., Carpenter, S. G., Wilder, M. E., Sebring, R. J. and Raju, M. R. (1989) Radiobiology of ultrasoft X-rays. III. Normal human fibroblasts and the significance of terminal track structure in cell inactivation. Radiat Res. 119:511–522.