EXTENDED ABSTRACTS

Proceedings of the 6th International Workshop/12th L. H. Gray Workshop: Microbeam Probes of Cellular Radiation Response

St. Catherine's College, Oxford, United Kingdom, March 29–31, 2003

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   The extended abstracts which follow this introduction present a summary of the proceedings of the 6th International Workshop/12th L. H. Gray Workshop: Microbeam Probes of Cellular Radiation Response, held at St. Catherine's College, University of Oxford, UK on March 29–31, 2003. In 1993 the 4th L. H. Gray Workshop entitled ''Microbeam Probes of Cellular Radiation Response'' was held at the Gray Cancer Institute in Northwood. This was organized by Prof. B. D. Michael, Dr. M. Folkard and Dr. K. M. Prise and brought together 40 participants interested in developing and applying new microbeam technology to problems in radiation biology (*1*). The workshop was an undoubted success and has spawned a series of subsequent workshops every 2 years. In the past, these workshops have been highly successful in bringing together groups interested in developing and applying micro-irradiation techniques to the study of cell and tissue damage by ionizing radiations.
   Since the first microbeam workshop, there has been a rapid growth in the number of centers developing radiobiology microbeams, or planning to do so, and there are currently 15–20 worldwide. Much of the recent research using microbeams has used them to study low-dose effects and ''non-targeted'' responses such as bystander effects, genomic instability and adaptive responses. The goal of the 6th workshop was to build on our knowledge of the development of microbeam approaches and the application to radiation biology in the future. In a meeting stretching over a 3-day period, over 80 participants reviewed the current state of radiobiology microbeam research worldwide and reported on new technological developments in the fields of both physics and biology.

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Session I

Chair: B. D. Michael

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The Future of the GCI Microbeams

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The Gray Cancer Institute (GCI) has been involved in the development and application of microbeams of ionizing radiation in a radiobiological context since the early 1990s. We now have two fully operational micro-irradiation facilities, with a third facility under construction. One facility makes use of an existing 4 MV Van de Graff accelerator to generate collimated beams of singly or doubly charged particles, and it has been used for cell irradiations for over 7 years (*1*). The second facility uses low-energy (ultrasoft) X rays, focused by diffraction optics to a sub-micrometer spot; it has been operational for 4 years (*2*).

The list of possible applications for microbeams in radiobiology continues to grow and diversify. Many of the experiments envisaged currently exceed or at least stretch the capabilities of many operating or planned facilities. From the outset, the GCI microbeams have been in a constant state of evolution. A number of aspects of our microbeams' performance continue to be scrutinized, and where possible, improvements are implemented. Areas where performance gains are being sought include increasing the cell throughput, increasing the targeting accuracy, increasing the penetration of the radiation, and the implementation of non-UV-radiation methods for target visualization. Developments pertinent to imaging cells are presented elsewhere in these Proceedings, but the other aspects are discussed in greater detail below.

Increasing Targeting Accuracy and Cell Throughput

It was evident from a series of critical tests and evaluations that in the case of our charged-particle microbeam, both the accuracy and the speed were being compromised by the sample positioning stage (*3*). An identical stage (Märzhäuser, Germany) is used on our X-ray microprobe and is similarly restricted in targeting accuracy, but in that instance, cell throughput is currently limited by the X-ray dose rate. Our measurements showed that position errors up to 3 μm were observed when cells located at the edge of the microscope field of view were moved to the center (i.e. to the irradiation position). Where necessary, this error can be reduced to about 1 μm using a "two-pass" system where each cell is imaged twice, but this takes longer and the extra UV-radiation exposure is undesirable. The error is due to the mechanical properties of the stage, which uses a stepper-motor and lead-screw arrangement in an open-loop configuration (i.e. no position feedback). Further investigations revealed that other commercial biological microscope stages, even those that use position encoders, offer little improvement on this level of performance. Good accuracy is achieved by locating the stage position encoders and linear bearings close to the sample, but this is not possible using stages designed for trans-illumination, which are of an "open-frame" design. However, it is also true that commercial stages of this type cater for a range of loads and viewable areas that are significantly in excess of our requirements for microbeam applications. By restricting the sample viewing area to, say, 25 mm², it is possible to design a stage that is both more compact and lighter than those available commercially, which improves rigidity, reduces the inertia of the moving parts, and places the position encoders closer to the sample.

With this in mind, we have developed a new stage that uses two voice-coil d.c. motors (BEI Technologies Ltd.) and two linear encoders with ±100-nm position sensitivity (Jena Numerik) to move and provide closed-loop position control of the stage over a 25-mm² area. By switching to d.c. motors, the software communication step with the stage is significantly shortened; since the new stage is lighter, it is capable of much faster accelerations and shorter "settling times". With the old system, it typically takes about 400 ms to irradiate each cell (~9000 cells per hour), of which 320 ms is accounted for by stage movement. The new stage is much faster, since cells separated by a few hundred micrometers can now be positioned in about 40 ms. Allowing for software communication and mechanical settling time, an average cell throughput of about 120 ms per cell (~30,000 cells per hour) is achieved. Our tests of the stage position accuracy indicate that an accuracy and reproducibility of less than 1 μm have been realized. However, we have also discovered that the overall accuracy is now limited by other mechanical factors, such as the stage support, since it can shift by about 1–3 μm, due to the momentum transferred by the rapidly moving stage. Given this constraint, we are currently optimizing the operating conditions to achieve the best compromise between accuracy and speed, but ultimately, further development of other mechanical aspects of the microbeam will be necessary to exploit the full capabilities of the new stage.

Increasing the Penetration of the Radiation

There is considerable interest in applying micro-irradiation techniques, not just to isolated cells, but also to multicellular samples, tissues and indeed organisms. While we have been successful in using a tissue explant model with our microbeams (*4*), the radiations available to us are only able to penetrate a few cell layers at most. Indeed, using our soft X-ray microprobe with 1.5 keV Al_K X rays (currently the highest X-ray energy routinely available to us), only the first cell layer can be targeted usefully. We are evaluating and, where appropriate, implementing a number of approaches designed to increase the penetration of both the particle and X-ray microbeams. In the case of the charged-particle facility, our immediate strategy is to reduce the energy lost by the particle before it reaches the cell. Most energy is lost in the 18-μm-thick scintillating layer that is used in our particle detection system. The thickness has been chosen to provide the minimum signal necessary for the scintillator light generated by the passage of a charged particle to be reliably discriminated by a photomultiplier tube immediately above the dish. More efficient, low-noise photomultiplier tubes are now available, but they can only be exploited in very low ambient light conditions. We are therefore redesigning aspects of our facility to cater for this. One useful change that will be made is to enable the scintillator light to be collected through the microscope objective, rather than the current arrangement, which requires the photomultiplier tube to be swapped with the objective at the irradiation step. With the new system, switching between viewing and detecting cells will be achieved simply and rapidly using a flicking-mirror arrangement.

A longer-term strategy is to consider improving the particle accelerator. Currently, the highest-energy ³He particles we can generate have an energy of 5.7 MeV, corresponding to a range in tissue of about 40 μm (allowing for losses in the detector). The maximum energy is limited by the performance of the final bending magnet, rather than the accelerator, so one option is to install a new magnet. This would enable an increase of up to 40% in the maximum energy and would achieve about 100 μm penetration in tissue for ³He particles. We have also conducted a feasibility study to evaluate the possibility of building a new microbeam, based on a 5 MV Tandetron sited at GCI. This would offer the ability to generate ³He particles with a penetration of about 290 μm and protons that can penetrate up to 1.2 mm in tissue.

With regard to our X-ray microprobe, our current facility was initially designed and optimized for 0.28 keV C_K X rays (*2*), but it has recently been modified so that 1.5 keV Al_K X rays can also be generated and focused, providing a modest but useful increase in penetration. While the move from using C_K X rays to Al_K X rays is highly beneficial, there are a several reasons why it would be advantageous to also use 4.5 keV Ti_K X rays. For example, the 1/e attenuation for Ti_K X rays is 170 μm,

compared to 7 μm for Al_K X rays and just 1.9 μm for C_K X rays. This enables cells beyond the first cell layer to be irradiated when using tissue-like samples. Also, using Ti_K X rays leads to a reduced dose variation as the X-ray beam passes through the cell, and from a microdosimetric point of view, they are more like high-energy X rays. A focused beam of Ti_K X rays therefore provides a powerful method of delivering a low dose of low-LET radiation to a single cell, since it has all of the significant characteristics of high-energy X rays but is reliably constrained to the selected target (since, unlike particles, low-energy X rays are not appreciably scattered). Using focused Ti_K X rays, it will be possible to simulate the passage of a single electron track. A single electron will deposit about 1 mGy in a cell, which can be replicated by delivering about 600 (absorbed) Ti_K X rays in to a single cell.

To develop a microprobe that can focus Ti_K X rays, the decision was made to build a wholly new facility rather than modify our existing source. The new source, currently under construction, uses similar principles for X-ray generation, i.e. by focusing energetic electrons onto (in this case) a titanium target. However, to achieve useful dose rates, much higher powers are required, such that a higher output electron gun and cooled target assemblies are necessary. Both the electron gun and the target assembly are being designed and constructed in-house. The target assembly makes use of a rotating anode (Rigaku, Japan) with a ferrofluid vacuum feedthrough and a hollow rotating shaft that allows coolant to be flushed through the titanium target. The electron gun uses a high-brightness lanthanum hexaboride filament and can be micro-aligned externally to maximize the output. Focusing will be achieved using a permanent annular magnet and soft-iron pole piece that has been designed using finite-element analysis modeling.

We have obtained and tested zone plate diffraction lenses optimized for Ti_K X rays, and these indicate an overall efficiency of about 4.5%, We are also considering several alternative methods for focusing higher-energy X rays (such as ''microstructured optical arrays''); these are discussed elsewhere in these Proceedings.

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The PTB Focused Microbeam for High- and Low-LET Radiation

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The PTB's ion accelerator facility comprises a 3.75 MV Van de Graaff accelerator and a cyclotron with maximum beam energies of up to 20 MeV for protons, 35 MeV for ³He ions, and 28 MeV for ⁴He particles (*1*). This range of ions and energies allows us to choose an LET between 3 keV/μm and 200 keV/μm, which covers almost entirely the range of naturally occurring radiation. In summer 2000, a beamline for radiobiological experiments was completed that is capable of targeting subcellular structures, cell nuclei in particular, with a counted number of ionizing particles.

In contrast to existing radiobiological microbeams (*2–4*), which use collimated beams, the PTB microbeam makes use of the focusing properties of magnetic quadrupole lenses. The unique ion-optical setup incorporates a bending magnet and two magnetic quadrupole doublets into the focusing system. This allows the experimental area to be located on the basement floor with the beam coming from above. The demagnification of the ion optical system is 20 in the axial direction and 7 in the radial direction. Beam spots with a diameter of 1.5 μm to 3 μm (fwhm) are routinely achieved.

Since the PTB microbeam uses focusing by magnetic fields, no particles scattered by any collimators occur at the target. This is particularly important for high-energy protons, which cannot be collimated effectively to micrometer dimensions due to their long range in matter. In addition, the beam current can be adjusted with the first slit arrangement without compromising the spatial resolution and the energy spread by slit scattering. Thus the energy spectrum has a negligible fraction of low-energy particles ($<10^{-3}$ of the total number) and the energy spread of the ions is very small, caused only by the energy straggling in the foil of the vacuum window. However, the first experiments showed an amount of ≈3% of particles with large spatial deviations from the nominal position. It has been proven experimentally that this effect was caused by the residual gas molecules in the beam line. An improvement of the vacuum reduced this effect to less than 1%.

The experimental area is equipped with an inverse microscope (Zeiss Axiovert100), an XY stage (Märzhäuser, Wetzlar, Germany) and a CCD camera (Spot, Diagnostic Instruments). The beam leaves the vacuum through a narrow aperture (∅ = 0.25 mm − 1 mm) covered with a Mylar foil (5 μm thick). Cells are located on a horizontal dish and are moved by the XY stage. Single-particle detection is performed with scintillating foils (BC400) between 10 μm and 35 μm thick and a photomultiplier tube (Hamamatsu R7400P), which is fixed on the microscope turret (*3*).

Before every irradiation, the cell dish is scanned with the CCD camera. Then a commercially available imaging program (ImagePro, Mediacybernetics) automatically analyzes the positions of the stained cells (0.5 μM Hoechst 33258). Depending on the cell preparation, between 95% and 100% of the cells are correctly recognized. This preparatory work typically takes 15 min. Thereafter, every cell is exposed to a chosen number of ions in an automated procedure that allows 20–50 cells per minute to be irradiated.

Earlier studies of cell positioning revealed systematic errors of the order of ±4 μm. This effect was due to mechanical imperfections in the XY stage. After a calibration with a laser interferometer, the accuracy of the cell positioning could be greatly improved to ±1.5 μm at present. This targeting accuracy has been verified for proton and ⁴He-ion beams by detailed studies with CR-39 track-etch detectors.

Cell irradiation started in autumn 2001. Since then the reliability and the convenience of the cell recognition and the experimental routines have been greatly improved, and up to 15,000 cells per day can be irradiated in routine operation. A laboratory room for biological preparations in proximity to the irradiation facility has been provided and equipped. Collaborations with several radiobiological research groups are in progress or have just started.

It is planned to increase the experimental capability of the facility using an electrostatic beam-scanning system. This allows the beam to be moved to the cells as long as the cell can be targeted within the area of the vacuum window. Thus the time-consuming procedure of mechanically moving to every cell can be avoided.

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System of Cell Irradiation with a Precise Number of Heavy Ions

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The use of heavy-ion microbeams provides a unique way to control precisely the number of HZE particles traversing individual cells and the localization of dose in an irradiated cell. In recent years there has been an increasing interest in the use of microbeams to study a number of important radiobiological processes in ways that cannot be achieved using conventional "broad-field" irradiation (*1–4*).

Energetic heavy ions (HZE particles) transfer their energy to biological organisms through high-density ionization along the particle trajectories. The population of cells exposed to a very low dose of high-LET heavy ions contains a few cells hit by a particle, while the majority of the cells receive no radiation damage. At higher doses, some of the cells receive two or more events according to the Poisson distribution of ion injections. This fluctuation of particle trajectories through individual cells makes it difficult to interpret radiological effects of heavy ions.

Using microbeams, we will be able to overcome this limitation. A microbeam can be used for selective irradiation of individual cells that can subsequently be observed to ascertain what changes occur to that cell and to neighboring unirradiated cells. The use of microbeams allows direct investigation of cell-to-cell communications such as "bystander effects", that is, radiation effects transmitted from irradiated cells to neighboring unirradiated cells. Furthermore, a microbeam with sufficient spatial resolution will be useful for analyzing the interaction of damages separately produced in an irradiated cell, the dynamics of intracellular process such as apoptosis, and the influence of the track structure of energetic heavy ions by means of highly localized irradiation of a part of a nucleus or cytoplasm.

Therefore, we have developed an irradiation system for targeting cells individually with a precise number of high-LET heavy ions to elucidate the radiobiological effects of exactly one particle (*5, 6*).

Heavy-Ion Microbeam Apparatus Used for Cell Irradiation

The cell irradiation system has been incorporated into the collimated heavy-ion microbeam apparatus (*7*), which was installed below a vertical beam line of the AVF cyclotron at the TIARA (Takasaki Ion Accelerators for Advanced Radiation Application) of JAERI-Takasaki. This apparatus was also designed to develop a novel radiomicrosurgery technique using local irradiation of living organisms with targeted heavy ions (*8*). The heavy-ion beams delivered from the AVF cyclotron are collimated with a set of apertures. Then the collimated beams are extracted into air through a microaperture on a 100-μm-thick tantalum disk perforated using an electrical discharge machining (spark erosion) method. The tantalum disk is attached to a gimballing arrangement and fixed on the micro-positioning X-Y stage, which is connected to the vacuum chamber with a flexible bellows, to achieve the alignment of the ion beams. A series of tantalum disks with various sizes of microapertures are ready to be used according to the desired spot size of local irradiation on the living organisms. The smallest microaperture, about 5 μm in diameter, was used for cell irradiation with a precise number of 13.0 MeV/nucleon ^{20}Ne^{7+} and 11.5 MeV/nucleon ^{40}Ar^{13+} ions.

Experimental Procedure

So far, two inverted optical microscopes (Nikon, TMD-300) are in operation in our cell irradiation system. One of the microscopes is installed below the vertical beam line in the beam room as an "online microscope" for cell targeting and for delivery of a certain number of heavy ions. The other microscope, which is called the "offline microscope", is used in the preparation room before and after irradiation for cell finding prior to the irradiation and for cell revisiting and observation during postirradiation incubation, respectively. A local area network connects these control systems, allowing the object database created at the offline microscope to be used by the cell targeting system. Details of the experimental procedure are described in another abstract from our group by T. Funayama *et al.,* "Effect of Single Argon-Ion Hit on Mammalian Cultured Cells".

Preparation and detection of target cells. Chinese hamster ovary cells (CHO-K1) are inoculated into the cell dish, the bottom of which is made of 100-μm-thick ion-track detector CR-39. The cell dishes are incubated for 2–6 h to let the cells attach to the CR-39; then the cells are stained with a fluorescent dye (CellTracker Orange, Molecular Probes) that uses green light for excitation. Positional data for the individual cells are obtained by fluorescence microscopy at the offline microscope in the preparation room before irradiation. The object database created at the offline microscope is shared by the online microscope cell targeting system. Just before irradiation, the medium is removed to make ions penetrate both the cells and the bottom of the cell dish. The cells are covered with a Kapton (8-μm-thick polyimide) film to prevent drying and microbiological contamination during the exposure to air.

Cell targeting and irradiation. The cell dish mounted on a sample holder is transported from the offline microscope to the online microscope in the beam room. The beam collimators and the whole optical microscope system are held together with an anti-vibration damper. Then the beam room is closed and no one is allowed to stay inside during irradiation to comply with radiation protection regulations. For this reason, all functions of this system, e.g. focus, lighting, objective and stage positioning, are controlled from the neighboring preparation room. Using the previously obtained object database, target cells are semi-automatically positioned exactly below the microaperture one after another. Then the object lens is replaced with a plastic scintillator (BICRON, BC-400) coupled to a photomultiplier tube assembly (Hamamatsu, R7400P–03, E5780) mounted on the online microscope turret. The collimated ions are detected with the photomultiplier assembly after passing through a target cell and the bottom of the cell dish. Energy spectra of collimated ions are measured with a multi-channel analyzer by analyzing scintillation pulses. The photomultiplier signal is large enough to eliminate the signal-to-noise ratio problems. This approach has another advantage in that the

detection process does not cause the divergence of the ion beams that can happen when using an ion chamber or a secondary electron foil upstream of the target. The number of ions penetrating the sample is counted with a constant fraction discriminator coupled to a preset counter/timer. Every irradiation is terminated by the action of beam shutter, which is controlled either by the preset counter/timer module or manually.

Prompt detection of ion tracks on target cells and revisiting during postirradiation incubation. Immediately after irradiation with 13.0 MeV/nucleon ^{20}Ne^{7+} and 11.5 MeV/nucleon ^{40}Ar^{13+} ions, the cell dish is refilled with medium, and the number of ion tracks penetrating the CHO-K1 cells is detected with prompt etching of CR-39 with alkaline ethanol etching solution at 37°C for 15–30 min. Usually, almost all the ion track pits are concentrated within a collimated diameter range. No significant effect of this etching treatment on cell growth was observed up to 60 h after irradiation. It is possible to revisit each irradiated cell repeatedly during postirradiation incubation according to the object database.

Conclusion

A method for irradiating individual cells with a high-LET heavy-ion microbeam has been established. Prompt detection of ion tracks provides us with accurate information about the spatial distribution of the delivered ions just after the irradiation. With this method, we can observe the number of ion hits and their positions on and around the target cells at the beginning of the postirradiation incubation of the cell samples. This method will be quite useful because the accuracy of irradiation information is important to the study of low-dose effects, especially the effects of exactly one particle. The effect of nuclear/cytoplasmic irradiation, bystander effects, and the influence of the track structure of high-LET heavy ions are being studied.

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Session II

Chair: U. Giesen

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First Experiments on Cells Using the Focused Microbeam at CENBG

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During the last few years, different groups have reported the development of focused microbeam facilities that are capable of delivering an exact number of particles to individual cells. Those systems are believed to constitute a powerful alternative technique to collimated microbeams, but to date, no results of radiobiology experiments using these beams have been published. Such a development was undertaken in 1998 at CENBG. The first version of this system is now fully operational, and the first experiments on cell lines started a few months ago. This setup is based on an existing focused horizontal microbeam, previously used for ion beam analysis under vacuum. For cell irradiation in air, a removable stage including a sample positioning system, an epi-fluorescence microscope, and a transmission detector can replace the usual top cover of the vacuum chamber. The advantage of this versatile system is to allow the switching from analysis under vacuum to irradiation in air within 2 h (*1*). To ensure a better preservation of cells during the irradiation procedure, dedicated vertical culture dishes have been designed to keep adherent cells fully immersed in medium. In this approach and for beam blanking purposes, the incident particle must be detected before it penetrates the culture dish, resulting in unavoidable beam straggling in any detector employed. On the other hand, since there is no collimator exit that can be used as an optical reference, localizing the beam in absolute mode requires a complex calibration procedure. This feature could be one of the main drawbacks of focused microbeams compared to collimated ones.

During the last 2 years, a large number of improvements have been made on the CENBG setup; the main effort has been put into the development of software to allow the control of the entire experiment in semi-automatic mode. Great attention has been paid to elaborate calibration procedures, including beam positioning and calibration of the relative movement of the sample stage within the microscope field. A dedicated cell culture protocol has also been developed. Numerous experiments have been performed either by using CR-39 particle track etching or by irradiating cells to demonstrate the reliability of our facility.

Control Software and Irradiation Procedure

The software used to control the instruments and all procedures was written on the basis of an object-oriented architecture using Microsoft Visual Basic© language. It includes several tools used for sample positioning, image acquisition, image processing (based on Image-Pro Plus 4.0, Media Cybernetics), and beam control. The calibration allows the determination of the absolute beam position and the calculation of coefficients correlating the three different coordinate frames (the cell dish frame, the microscope frame and the beam scanning frame). An automated irradiation procedure, based on a mosaic of microscope fields (up to 100) that covers the entire culture dish, has been designed. This software has been tested extensively in real conditions of irradiation. It was demonstrated that the entire irradiation sequence, including full calibration, cell localization and irradiation, can be performed at a rate of more than 2000 cells irradiated within 1 h. These tests were carried out with a steady beam passing through the 10-μm entrance collimator (*2*) of our gas transmission detector (3.5 mm thick, 10 mbar isobutane). A much

higher irradiation rate is expected for future experiments when the beam will be positioned on cells using the electrostatic scanning plates of the microbeam.

Targeting Accuracy

To assess the targeting accuracy, we employed a method described by the Gray Laboratory group and published in ref. (*3*). Using the beam scanning capability of our microbeam under vacuum, we produced arrays of circular patterns on CR-39 track detector foils (with diameter close to cell size) that were revealed after etching in concentrated KOH. Then the etched circles were used as an optical reference for the irradiation system that automatically calculated targeting positions halfway between two circles. Using this technique, 50 α particles were delivered on each target and the CR-39 foils were etched once again. Light microscopy permitted us to determine the beam spreading and the accuracy of the irradiation sequence in the case of a steady beam passing through the gas detector. The CR-39 foil was placed downstream at a distance of 200 μm from the Si_3N_4 exit window of the detector. We found that for these routine irradiation conditions, about 95% of particle tracks were localized within a circle 10 μm in diameter. The targeting accuracy, which reflects the capability of the system to align the target and the beam properly, was evaluated by measuring the distance between the actual pit and the expected position of this impact. This accuracy was evaluated to be ±2 μm. The performance may be improved by reducing the air gap between the exit window and the target position but at the price of a reduction in the safety of operation, more especially for the window integrity. This difficulty may be solved by moving the culture dish to an irradiation position as close as possible to the window and by moving it slightly away during the drive movement. This possible sequence is implemented in our control-command system, but the irradiation rate was found to be greatly reduced.

System Calibration

To ensure a reliable irradiation in terms of single event control and beam positioning, an online calibration procedure has been implemented in the control-command software. This method is based on the measurement of the transmission yield through a 10-μm circular collimator in air. To make this task easier, the collimator is automatically centered on the beam position by mechanical scanning. Since we have found that each time a culture dish is removed or placed on the system, there is a risk of moving the optical bench supporting the irradiation stage and then to generating positioning errors of a few micrometers, the system is systematically recalibrated after each manual operation on the irradiation stage.

The reliability of the single event control may be measured at any time by using a solid-state particle detector consisting of a PIN diode placed in air in front of the exit window of the gas detector. The diode working in coincidence mode with this detector allows the measurement of its efficiency. In routine conditions, this efficiency was found to be better than 99.5% and, as expected, was dependent on the gas pressure within the detector. In addition, with the 10-μm calibration collimator placed in front of the PIN detector, the transmission yield may be measured in the same conditions. This yield generally ranges from 85 to 98% as a function of the gas pressure and of the distance between the collimator and the exit window.

Cell Culture Conditions

To preserve the physiological status of cells during the irradiation, cylindrical polyethylene dishes (1 cm in diameter) were designed with the aim of keeping the cells fully immersed in medium. Cells attach on a 4-μm-thick polypropylene foil pretreated with an adhesive factor chosen according to the cell line. Different films were tested, and polypropylene was found to emit the lowest yield of blue light under UV excitation and thus to provide the best signal-to-noise ratio for the recognition of stained cells. Water-tightness is obtained by stretching the polypro-

pylene foil on the dish using a ring. The back face of the dish is closed by a 200-μm-thick circular glass microscope slide squeezed on a dry silicon joint by means of screws. By tightening the screws, the pressure within the dish can be carefully adjusted to obtain the lowest curvature of the culture support. This ensures uniform microscope focusing on the whole dish area during the irradiation procedure. In these dishes, a medium layer about 3 mm thick covers cells during irradiation. The non-cytotoxicity of the dish components, including the silicon joint, was assessed by scoring colonies after the cells had been maintained for more than 7 days within the dishes.

During the preliminary irradiation tests, human keratinocytes of the Ha-CaT cell line were employed. Usually cells are seeded several hours prior to irradiation in dishes and cell nuclei are stained with Hoechst 33342 dye at a concentration of 1 μ*M* 90 min before irradiation. When a large cell number was required for statistical reasons, we tested different cell concentrations and found that 1500 cells seeded in an area of about 5×5 mm² in the center of the dish was the best compromise.

Concluding Remarks

The CENBG beam line is still under reliability assessment. First experiments consist of comparison and cross-checking of survival curves with those obtained using other irradiation techniques including α-particle sources and broad-beam irradiation. Nevertheless, a research program dealing with induction of apoptosis by ionizing radiation and associated signaling mechanisms is going to be initiated. Different methods are planned to characterize radiation-induced apoptosis at the individual cell level: FT-IR microspectroscopy and fluorescent probes such as the GFP (green fluorescent protein). This *in vivo* labeling technique combined with live cell imaging allows the monitoring of the early phases of apoptosis.

From a purely technical point of view, the development of focused microbeams proved to be more complex than expected with regard to calibration procedures, reliability in routine conditions, and beam control at efficiencies close to 100%. Nevertheless, these systems appear to be the only alternative to collimated beams when a resolution better than 5 μm is required, the theoretical limit of collimator scattering being reached by the collimated microbeams in operation today. An adequate transmission detector still must be designed for light particles such as α particles in the MeV energy range. Gas detectors suffer from gas scattering even at very low pressure, resulting in a few percent of particles scattered outside the main spot. The reliability of secondary electron detection, with noisy detectors very sensitive to pressure fluctuation, still requires some improvement, especially if we consider the very few electrons emitted by classic window materials. Thin scintillator foils (less than 10 μm thick) have been used successfully when efficiently coupled to miniature photomultipliers working in coincidence mode. As an exit window, however, their use is limited to proton detection since the range of heavier MeV particles is too short.

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Progress Report on the INFN-LNL Horizontal Single-Ion Microbeam Facility

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In the past few years, a horizontal single-ion single-cell microbeam apparatus has been designed and installed at the INFN-LNL broad-beam radiobiology facility of the 7 MV Van de Graaff CN accelerator which delivers light-ion beams ($^1H^+$, $^2H^+$, $^3He^{2+}$, $^4He^{2+}$) in an LET range from 7 to 150 keV/μm (in tissue) (*1, 2*).

Recently, a number of specific elements of the facility have been improved and developed. In particular:

1. Different microcollimator devices with apertures down to 1–4 μm² (slit microcollimators or pinhole microcollimators) have been made and tested. The performance of the microcollimators and of the alignment system has been assessed in terms of the beam spectrum quality in air (full-energy peak/whole energy spectrum) as well as of the particle counts, measured at the position of the cells to be irradiated, with a silicon detector (EG&G SSBD, 300-μm-thick depletion region, 50-mm² sensitive area) and conventional spectroscopy instrumentation.
2. The best results have been obtained with a one-piece tantalum disc (200 μm thick) with a central hole 2–3 or 5 μm in diameter (*2, 3*). In the best alignment condition, using 3.5 MeV (2.9 MeV in air) protons and 8.9 MeV (5.9 MeV in air) $^3He^{2+}$ and 10 MeV (6.6 MeV in air) $^4He^{2+}$ ion beams, the full-energy peak reached 80% (for 5-μm pinhole) and 70% (for 2–3-μm pinhole).
3. With a beam spot with an area of 3–20 μm², a particle count rate as low as one ion per second is achievable.
4. A high-resolution and high-sensitivity custom-made cooled CCD camera (DTA, Italy) has been developed to measure the collimated beam profile and exact beam position in air. It has been mounted close to the cell holder (*2*) in such a way that the pixel matrix is at the same distance from the microcollimator as the cell sample to be irradiated. The cooled CCD camera is constructed without any optics or shutter, but with a 3-μm-thick havar window above the pixel matrix. This matrix is an array of 2184 × 1472 pixels; every pixel is 6.8 × 6.8 μm².
5. A dedicated software program has been developed by using the LabView 6.0 software package (National Instruments) to acquire the CCD pixel signals and to analyze the spatial distribution of the particles to determine the exact position of the particle beam in air (the centroid of the spatial particle distribution).
6. The semi-automatic system for cell recognition, positioning and revisiting, based on micro-positioning stages (Physik Instrumente) and an inverted phase-contrast optical microscope (Olympus), has been modified and reinstalled to include a specific phase for the positioning of the cooled CCD camera on the beam for the measurement of the microcollimated beam profile (spatial particle distribution centroid).
7. High-precision actuators (MARPOSS) have been mounted along the helicoidal guided translation system, allowing the movement of the biological sample from the horizontal position under the microscope objectives to the vertical one in front of the microcollimator on the ion beam to increase the positioning accuracy (*1, 2*).

A dedicated software program (CELLView) has been developed by using the LabView 6.0 package to control every step in the cell irradiation protocol (*3*). CELLView allows precise control of the following cyclic operation:

1. The remote-controlled movement of the cell holder from microscope position to beam or to cooled CCD position, respectively, and vice versa.
2. Semi-automatic cell recognition and coordinate logging: Under the inverted phase-contrast optical microscope, two fiducial markers placed near the cell holder are localized and their coordinates are logged. One of these markers is taken as the origin of the reference system for the coordinates of all single cells. Cell visualization is done through an optical microscope equipped with a color CCD camera (Philips), without any fluorescent staining or UV light. The X-Y coordinates of the cell are logged and the cell images are acquired and transferred to a dedicated PC.
3. Cell holder positioning on the microcollimated beam: Another CCD camera mounted close to the microcollimator assembly is used to relocate the fiducial markers after the sample platform is moved from microscope to the beam position. Every cell's X-Y coordinates are then corrected using the software on the basis of the "new" values of the reference markers.
4. Correction of the cell coordinates for the exact beam position in air: the exact beam position is determined relative to the fiducial markers with the custom-made cooled CCD camera used as beam monitor (*2, 3*) by means of software developed to acquire the microcollimated beam profile in air and to calculate the centroid of the particle spatial distribution. Every cell's X-Y coordinates are then corrected for the (centroid) beam position and saved in the data file.
5. Cell irradiation: Every cell is automatically positioned in the microcollimated beam during a run by recalling the logged coordinate values.
6. Cell revisiting: After irradiation, the sample platform is again positioned under the microscope for cell revisiting and image acquisition to check the accuracy of the positioning.
7. A user-friendly interface has been created to input the necessary data (in the semi-automatic cell recognition phase) and to control and display all the information for the different cyclic procedures.

A new stainless steel petri dish has been designed and constructed to obtain a very thin cell holding depth of about 20 μm to allow the particle crossing the cell dish (containing cell population and culture medium) to impinge on the silicon detector used for detection of the single ion (*2*). The bottom and the cover of such a cell vessel consists of thin (7 or 52 μm) Mylar foils. Alternatively, a very thin CR-39 foil (about 50 μm thick) can be used in place of the Mylar on the petri dish bottom to log every particle impact point for subsequent postirradiation analysis of ion hits and cell position.

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Single Particle Irradiation System to Cell (SPICE) at NIRS

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Introduction

Recent evidence of bystander effects gives a strong impetus for radiation research at low doses and low dose rates to examine the mechanisms of radiation actions and to estimate risks for radiation protection (*1–4*), though little is known about the mechanism(s) of the bystander effect.

Keeping these results in mind, and with our special interests in radiation protection as it relates to radon exposure in the environment (*5*) and cosmic-radiation exposure in space at the ISS (International Space Station), we have started a project to build a microbeam facility called the Single Particle Irradiation System to Cell (SPICE). Here we describe the facility that is under construction.

Materials and Methods

Accelerator. The accelerator used to produce protons and helium ions for this project is a tandem and single-ended accelerator with a solid-state high-voltage power supply, Tandetron (HVEE, High Voltage Engineering Europe Ltd.), which was installed in 1999 for our PIXE (Proton Induced X-rays Emission) study. It consists of an off-axis Duoplasmatron ion source, an upper-stream low-energy slit stabilization magnet, a stable high-voltage power supply, and an upper-stream high-energy slit stabilization system. The performance of the accelerator meets the requirements of microbeam applications (micro PIXE) well. It achieves a terminal voltage ripple of about 16 Vp at 1.25 MV, its stability is around 18 V/3 h at 1.881 MeV, and the H^+ beam brightness is about 3.9 Am^{-2} rad^{-2} eV^{-1}. Two kinds of particles, 3.4 MeV H^+ and 5.1 MeV He^{2+}, are available.

Beam transport system. The existing PIXE beam ports were built at two ends of the horizontal beam ports from the accelerator. A vertical beam port was installed by branching it from one (longer) horizontal beam port through a 90° bending magnet. An electrostatic beam deflector was placed upstream adjacent to this bending magnet. The vertical port after the bending magnet consists mainly of a vacuum pump, an object slit and a triplet Q-magnet, directed to the irradiator (described below). The vertical beam port was installed together with the irradiator in a cradle (inner frame) as a single solid structure as a whole, which was hung on a rigid structure (outer frame). This special structure of the frames will enable the vertical beam port to be relatively insensitive to unavoidable environmental vibrations.

Microbeam. One of the more difficult parts of creating a microbeam is the design, construction and alignment of the final collimation of the beam. The triplet Q-magnet (Oxford Microbeam Ltd.) which was installed was manufactured according to the site requirement that the distance between the object slit and the focus point should not be greater than 320 cm. It was designed to achieve a focused beam spot smaller than 2 μm in diameter. However, for this new type of collimation, little is known about how to identify this size of beam at an extremely low fluence rate. Much effort and experience are necessary for this.

Irradiator. The irradiator consists of a dish holder positioning system, a beam detector, and a microscope system. The collimated particle comes out the exit window of the beam port up into the air and targets the cell dish. Positioning is done using light from a thin scintillator, produced by the bombardment of ionizing particles, which is placed at the bottom of the cell dish and observed by a microscope system. The cell dish is mounted on an X-Y stage, the movement of which is driven by a Voice Coil motor and controlled by a PC. The irradiator that is attached to the straight-through beam port is also equipped with a channel plate-based image intensifier followed by a CCD camera.

Acquisition of image data. The light source, a mercury vapor ultraviolet epi-fluorescence module, is used to illuminate a target of a cell. The imaging software obtains the coordinates of the target and then registers them for subsequent irradiation. After an image is captured, other software operates for precise positioning of the X-Y stage relative to the focus point of the microbeam such that a particle irradiates the registered target precisely.

Control of number of particles. The number of particles used for irradiation is controlled within around 1 μs by a PC, from the signals from the ion detector, by triggering the electrostatic ion deflector at the position upstream adjacent to the 90° bending magnet. The detector is a thin scintillation counter that is embedded in the revolver of the microscope, the position of which is replaced by that of the object lens for image capture.

Position resolution required. The goal is to achieve a position resolution of less than 2 μm, including all relevant factors such as coordinate acquisition of the target, precise positioning of the X-Y stage to the beam focusing spot, and beam scanning operation (optional at present).

Performance of speed of the system. An effort has been made to make the irradiation time as short as possible from data acquisition to repeated irradiation. At present our estimation of best performance is around 2000 cells/h.

Irradiation cabin. A workbench platform is built at a height of 460 cm from the floor on the outer frame structure with a lift for the platform. The platform is for operators to adjust the modules such as the triplet Q-magnet, the beam monitor, the controller of the cell dish stage, the particle detector, and electronics for users to manipulate the sample dishes.

Discussion

Experience in other institutions (*6–8*) suggests that the construction of the facility is only a start toward achieving estimated and expected performance, and that the actual achievement of irradiation with particle beams of micrometer size requires a trial-and-error process of tuning and adjustment of the microbeam. First, for the identification of the position of the beam spot at the cell dish, two optical devices of different sensitivity in light intensity are prepared for rough and fine adjustment. The rough adjustment of the beam size from a few hundreds of micrometers down to a few micrometers is carried out with an optical device of low sensitivity under the high fluence rate of ionizing particles. It is followed by a fine adjustment down to 2 μm with the optical system described above (of high sensitivity) using a low fluence rate of the particles. This is our first trial-and-error process. Our second trial-and-error process is to scan this beam spot to irradiate cells located at different positions in the same dish.

Several types of cultured mammalian cells that attach to the bottom of a cell dish are available for microbeam experiments. As others have suggested, however, other types of cells that grow in suspension in medium, such as lymphocytes, are also important as samples for microbeam irradiation. More study and some technical innovations are necessary for this application, some of which are under investigation in our group.

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First Irradiation Experiments with Living Cells at LIPSION

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We report here on the development of an irradiation platform for living cells at the high-energy ion nanoprobe at the LIPSION Laboratory, Leipzig, Germany, and on the first experiments with living cells that were carried out in the course of studies of radiation-induced bystander effects using a focused ion beam (*1*).

The LIPSION Nanoprobe Laboratory

The high-energy ion nanoprobe LIPSION at the University of Leipzig has been operational since 1998 (*2*). The ultrastable single-ended 3.5 MV Singletron™ accelerator supplies a H^+ or He^+ ion beam. The beam can be focused by a magnetic quadrupole lens system down to below 50 nm in diameter for the low-current mode. A magnetic scanning system moves the focused beam across the sample according to the desired number, width and velocity of the position steps. Thus we are able to apply a selected number of ions to each target position.

For the cell preparation and analysis procedures, we have established a cell culture laboratory adjacent to the accelerator laboratory.

Irradiation Platform and Mini-petri Dishes

The irradiation platform consists of a tunnel with a rectangular cross section which can be attached to a CF160 flange on our target chamber. Bellows allow for fine adjustment of the tunnel from the outside. The side facing the ion beam is equipped with an ion exit window made of Si_3N_4 that is 1 mm × 1 mm in area and 100 nm thick (called frames because the windows are framed by 200 μm silicon). This exit window bends toward the vacuum side of the beam line but withstands a pressure difference of one atmosphere. A mini-petri dish holder can be slid into the tunnel with a sufficient gap between the exit window and the bottom of the petri dish and is subsequently moved toward the exit window, ensuring a minimal air gap. The mini-petri dish is a conventional 35-mm-diameter plastic dish with a Si_3N_4 window at the center whose dimensions are 2 mm × 2 mm and a thickness of 200 nm. The particle detector is placed downstream immediately after the cells.

Adhesion Tests

We carried out adhesion tests with EaHy 926, a hybrid cell line (fusion of HUVEC and epithelial cell line A459) grown in enriched DMEM (*3*). We used about 0.25 million cells/ml and added 2 ml cell-containing medium to the entire bottom of the mini-petri dish. In this way we seeded an average of 2000–4000 semi-confluent cells on 4 mm². Cells that did not adhere were taken up together with the medium with a micropipette and were counted in a counting chamber. We found that up to 50% of the cells adhere after 14 h equally on the petri dish and on the Si_3N_4 frame. Using trypan blue staining, we determined that most of the cells that did not adhere were dead. Similar results were obtained with polyethylene terephthalate (PET) foils that were 0.9 μm thick; however, these were abandoned because they wrinkle easily. The ability to adhere was also studied with fluorescent Hoechst 33258 stain. The cells were incubated with 1 μ*M* Hoechst stain 33258 for 1 h and then put into Hepes medium.

Medium Removal, Vertical Positioning, Short-Term Survival, and Migration Tests

We are using a horizontal beam line and therefore must bring the mini-petri dishes to a vertical position for irradiation. Since we have the particle detector behind the cells, we must remove as much medium as possible, at least at the position where we plan to irradiate. Using a micropipette, we removed the medium and washed the cells twice with PBS buffer so that a thin film remained in the center of the window while a significant meniscus remained at the edges of the frames. About 95% of the cells survived the following procedure as checked by fluorescein diacetate (FDA) and propidium iodide (PI) staining: medium removal, vertical positioning and sham irradiation for 15 min, horizontal repositioning, and restoration of the usual medium level. Extensive studies of the accuracy of the cell positions before and after this procedure were carried out. Images of stained cells (Hoechst 33258) were recorded with a digital camera, and it was found that cell positions were unchanged within the resolution of the digital camera (2000 × 1400 pixels), apart from those cells that did not survive and floated away. Thus the immobility of the cells is sufficient. The normal situation encountered was that a single cell was totally missing after the procedure and might have floated away from the field of view. Occasionally, a new cell that might have floated into the field of view from outside appeared after the whole procedure.

Irradiation of Living Cells

An area of 540 μm × 540 μm close to the upper edge of the 2 × 2-mm Si_3N_4 frame that was roughly centered was irradiated with 2.25 MeV protons in the scanning transmission ion microscopy (STIM) mode with the following parameters: We used 250 × 250 pixels, and the beam scanner was advanced by one pixel after a single proton was detected. In this way a rectangular grid with 2.16 μm spacing with exactly one proton per point was produced. This allowed us to get a STIM image of the cells, albeit with less resolution than would normally be achievable (with smaller pixel size).

The energy loss from the ion exit window, the bottom of the mini-petri dish, the air gap between both (estimated to be less than 100 μm), and about 4 mm air in front of the particle detector was calculated to be roughly 140 keV using the SRIM code (SRIM-2003; http://www.srim.org). The cells received on average about 100 protons. The linear energy transfer (LET) for 2.25 MeV protons is of the order of 15 keV/μm. With an estimated cell thickness of 5 μm (averaged over cytoplasm and nucleus), each cell received an energy of about 5–10 MeV. Using the above numbers, we thus estimate that each cell received a dose of about 0.5 Gy.

We subsequently stained the cells with FDA and PI and checked for survival at 15 min and 17.5 h after irradiation; a very large number of cells survived.

However, further experiments are necessary to extract significant in-

formation on cell survival. In addition, further end points like interleukin 1α, β₁-integrin and ROS will be examined because ^{60}Co γ irradiations showed that these are sensitive indicators of the response of cells to ionizing radiation (4). We expect similar sensitivity for proton irradiation, although it is unclear whether we can reach the detectable threshold to visualize such changes.

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Development of a Charged-Particle Microbeam for Single-Particle Subcellular Irradiations at the MIT Laboratory for Accelerator Beam Applications

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Introduction

A charged-particle microbeam for single-particle, subcellular irradiations is nearing completion at the Massachusetts Institute of Technology Laboratory for Accelerator Beam Applications (MIT LABA). The microbeam apparatus includes an electrostatic accelerator, horizontal beam tube, a 90° bending magnet to render the charged-particle beam vertical, and a dedicated endstation for biological irradiations. The entire apparatus is less than 4 m long, so the microbeam can be completely housed in a single room. The He^{++} or H^{+} charged-particle beam is delimited using either a slit or a single-hole collimator assembly. Particle counting is performed below the cell dish with a plastic scintillator in combination with two photomultiplier tubes counting in coincidence mode. Control software, developed in-house, manipulates all aspects of the hardware including the accelerator, beam-line components, vacuum systems, and all subsystems of the biological endstation.

Accelerator and Beam Line

The microbeam uses a 1.5 MV single-stage electrostatic accelerator that is capable of generating a variety of particle types and charge states. The accelerator, designed by Newton Scientific Inc. (Cambridge, MA), is very compact: only 1.8 m long and 0.77 m in diameter. The accelerator comprises an accelerating column that contains the accelerating tube, the high-voltage power supply, and the high-voltage terminal assembly including the ion source and associated electronics. The ion beam is continuously injected at low energy (15–30 keV) into the accelerating tube from the radiofrequency ion source located in the high-voltage terminal. The beam is accelerated and focused by the electrostatic field of the accelerating tube and will attain a final maximum energy of 1.5 MeV (single-charged ion) or 3.0 MeV (doubly charged ions).

The particle beam emerging from the accelerator travels down a 1.2-m-long beam tube. This beam tube houses X-Y steering plates for beam deflection and a magnetic quadrupole triplet. In the microbeam's original configuration (a horizontal beam), this magnet was designed to provide a highly focused (1 μm) beam spot in vacuum for proton-induced X-ray emission (PIXE) and other surface analysis methods. Since a vertical beam was considered optimum for biological experiments, however, the focusing capability of the triplet is not used in the present configuration of the microbeam. Instead, this magnet is used simply to defocus the ion beam somewhat prior to its entry into a 90° bending magnet. The bending magnet serves two purposes. First, the ion beam now becomes vertical, which allows cell dishes to be positioned in the horizontal orientation. This is desirable if irradiation times are long and require some amount of medium to remain on the cells. Second, since the bending of ions in a magnetic field is a function of the charge on the ions, the magnet allows us to selectively transport the ions of choice into the vertical orientation. For instance, for the generation of helium beams, the magnet ensures the transport of 3.0 MeV He^{++} ions to the cell irradiation endstation and the rejection of 1.5 MeV He^{+} ions that would result in the detection of a particle (through interaction with the plastic scintillator located below the cell dish) but only partial or no cell irradiation due to the limited range of these particles.

Cell Irradiation Endstation

Subsystems of the cell irradiation endstation are housed in a 49 × 49 × 31-cm³ light-tight box mounted immediately above the 90° bending magnet. The cell irradiation endstation comprises the following subsystems: a collimator or slit assembly to delimit the beam, a two-dimensional motorized stage, a plastic scintillator, light guide and two photomultiplier tubes for particle counting, a specially designed cell dish, UV objective with z-motion for focusing, dichroic mirror, and a CCD camera for cell visualization. A light source is mounted on the outside of the endstation box; light passes from the source through a 3.2-cm-diameter tube to interact with the dichroic mirror. The deflection plates, motorized stage, photomultiplier tubes, light source, and CCD camera are interfaced with a PC for computer control of all aspects of cell irradiation.

Slit and Collimator

Beam delimiting is performed using either a slit or a single-hole collimator. The slit is a laser-drilled aperture (Lenox Laser Inc, Glen Arm, MD) 1.8 μm wide and 1 mm long drilled in 45-μm-thick stainless steel and glued to a 9.5-mm-diameter mount. This mount can be removed and the collimator assembly installed in its place. The collimator is made from 285-μm-diameter fused silica tubing with a 1.5-μm-diameter bore. Collimators are cut from the tubing in lengths of approximately 1 mm and inserted into a mounting assembly that also holds the single-particle counting apparatus (light guide, plastic scintillator and two photomultiplier tubes). Efforts are under way to characterize the "beam size" generated by the collimator in its mount using track-etch detectors (CR-39

and LR-115). Characterization of the radiation pattern produced by the slit has been performed using specially fabricated radiochromic film and demonstrates a beam width of approximately 3 μm.

Particle Counting

The particle detection system is located between the collimator and the cell substrate. This configuration allows detection of particles regardless of the thickness of medium covering the cells and in addition permits experiments in which it is desirable to have the particle stop within the cell. However, given the relatively low energy of the ion beam, a very thin transmission type detector is necessary to minimize energy loss of the particles to a few hundred keV so that sufficient residual range remains for irradiation of the cell through the cell dish substrate. A thin plastic scintillator (Alpha Spectra, Inc., Grand Junction, CO) has been chosen since it has a very fast decay time and is available in 5-μm thicknesses. To minimize false positive and negative rates in the thinnest possible scintillator, coincidence detection of scintillator photons has been adopted. Experiments performed to optimize light collection led to a design using a Lucite light guide into which two photomultiplier tubes are positioned. The scintillator is optically coupled to a slight inset in the light guide using optical cement, and the photomultiplier tubes are coupled to the light guide using optical grease. The light guide, optical cement, scintillator and optical grease all have similar indices of refraction. Signals from the photomultiplier tube are fed first into pre-amps and amplifiers, then into a timing single-channel analyzer, and finally into a coincidence analyzer. Investigations performed offline with a low-activity α-particle source have demonstrated that total counting efficiency can be improved by coating the light guide with a reflective paint and by other strategies to enhance the coupling of the photomultiplier tubes with the light guide and scintillator. Efficiencies of 96% have been attained, and work is under way to improve this further.

Session III

Chair: M. Folkard

Gray Cancer Institute, Northwood, United Kingdom

First Experimental Experience with the PTB Microbeam

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Introduction

Microbeams are essential research tools to investigate radiation actions and their mechanisms in the low-dose range down to some mGy. Among these are the induction and the repair of DNA double-strand breaks, chromosome aberrations when using the PCC technique, inducible repair [or hyper-radiosensitivity (HRS) to very low doses], and bystander effects. Several years ago, the Physikalisch-Technische Bundesanstalt (PTB) in Braunschweig and the University of Göttingen started a cooperative effort to establish a microbeam facility for radiobiological research at the accelerator facilities of the PTB. The full details of the physical setup of the PTB microbeam are given in an accompanying abstract by Greif *et al.*

Design of the Cell Dish

The focused microbeam has a downward direction, necessitating a specific construction for the cell dish and of the cell preparation. The charged particles, which must leave the beam line through a thin window in a downward direction, penetrate the foil (1.5 μm to 25 μm thick depending on the type and energy of the particles) to which the cells are attached. To minimize energy and angle straggling of the charged particles and to keep the cells under physiological conditions, the foil (with the attached cells) forms the front entrance of a flat cylinder (8 or 11 mm in diameter) surrounded by a 1-mm-high wall made from a specific medical stainless steel and closed by a 0.13-mm-thick cover glass at the exit front (12 or 15 mm in diameter). This flat cylinder is filled with buffered growth medium and allows a strong and bubble-free adhesion of the cover glass to the steel rim. For at least 1 h, no loss of moisture was observed. The plane parallel fronts of the flat cylinder allow an accurate visualization of the cells or their nuclei through the 1-mm-thick medium layer when using an inverse epifluorescence microscope. The stainless steel cell dish with an outer diameter of 20 or 25 mm is fixed in an aluminum ring on the X-Y stage.

Materials and Methods

In the first experiments, the cell dishes were covered with a biofoil (25 μm) especially prepared for cell adhesion. By using a specific glue, covering of the dishes with foils and sterilization were performed in one step at a temperature of 140°C. The dishes were ready for use without any further procedure. Freshly harvested, nearly confluent CHO-AA8 Chinese hamster cells were seeded in the center of the dish by pipetting 3.5 μl of the cell suspension onto the foil to give a final concentration of 200 to 300 cells on an area of 2 to 4 mm². The cells were allowed to attach overnight in a humidity chamber to avoid desiccation. The next morning 60 μl growth medium was added. About 2 h before the irradiation, the cells were incubated in growth medium with 0.5 μM Hoechst 33258. After 1 h of staining, the medium was removed, the cells were washed, and the medium was replaced with a 20 mM Hepes-buffered growth medium. After the scanning and irradiation, the cells were trypsinized and seeded into culture flasks for colony formation.

Further experiments were performed using confluent immortalized human skin fibroblasts (MRC5) and confluent primary human skin fibroblasts (HSF2) for colony formation. Because of the much lower plating efficiency of the HSF2 cells (≈10% compared to 60 to 80% for CHO-AA8 and MRC5 cells), a 3.5-μl HSF2 cell suspension was pipetted in the center of the foil to give about 10³ closely neighboring cells within an area of 2 to 4 mm².

The three different types of cells were irradiated with 50, 70 or 100 protons with an energy of 1.9 MeV and an LET of 16 keV/μm in the cell nuclei.

Results and Discussion

The results obtained with the CHO-AA8 cells show a clear dependence of the surviving fraction on the number of protons per cell nucleus. However, the statistical fluctuations were unacceptably high. It was suggested that the attachment of CHO-AA8 cells was not strong enough to resist their removal from the foil during the washing procedure before the trypsinization. Since fibroblasts are known to exhibit a stronger adhesion to surfaces, we repeated the experiments by using immortalized human fibroblasts (MRC5). Again, we obtained reasonable surviving fractions, with reduced but still unacceptable statistical fluctuations. In the third series, we used primary human fibroblasts (HSF2) and obtained a statistical fluctuation of the surviving fractions within an acceptable standard deviation of less than 20%.

These results of the first experiments with cells of three different cell lines at the PTB microbeam were reasonable results and were in agreement with comparable studies at the Gray Laboratory. It has been found that the attachment of cells to the biofoil is a critical cell-dependent parameter that must be determined for each cell line. Experiments are in progress to investigate the effect of covering the biofoils and the Hostaphan foils with Cell Tak or polylysine to improve the attachment of the cells.

The Munich Microprobe Setup for Single-Ion Irradiation of Cells

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The ion microprobe SNAKE [Superconducting Nanoscope for Applied Nuclear (Kernphysikalische) Experiments] provides focused ion beams at the Munich 14 MV tandem accelerator (*1*). It is used for various experiments like hydrogen microscopy (*2*), high-resolution scanning transmission ion microscopy, and materials modification on a microscopic scale (*3*). Operating in single-particle mode, 95.4% of the ions are delivered within 1.0 μm in the X direction and 260 nm in the Y direction under vacuum conditions. The different ion species and energies available from the 14 MV tandem accelerator offer stopping forces on water from 2 keV/μm (25 MeV protons) to several MeV/μm (e.g. 100 MeV ^{32}S). Therefore, SNAKE is an ideal instrument to use in a variety of cell irradiation experiments. An irradiation setup for upgrading SNAKE to a single-ion irradiation facility has been designed and is now under assembly.

The ion beam, focused by the superconducting multipole lens of SNAKE, is transported under vacuum conditions through a flexible coupling to a beam exit nozzle. There the horizontal beam enters the atmosphere by passing a 1-mm bore covered with a 6-μm Mylar foil. The cells to be irradiated are plated onto a thin foil in a specially prepared container positioned in the focal plane of the ion beam. To observe and align the cells, an inverted microscope (Axiovert 25, Zeiss) was purchased and adapted to the existing setup.

Since the beam exit nozzle can be moved both laterally and along the beam direction, it is possible to insert a microscope condenser for transmitted light illumination with phase contrast. In doing so, the cells can be visualized without using staining techniques that might disturb normal biological processes.

The irradiation experiments will be done with single, counted particles. This beam preparation is carried out using an electrostatic deflection system powered by a fast high-voltage MOSFET switch. The successful operation of this facility was proven by generating regular irradiation patterns consisting of etched tracks in glass plates. For particle detection during the cell irradiation experiments, a detector on the microscope turret stops the ions which have passed through the exit window, the cell container foil, and the irradiated cell.

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Status of the GSI Microbeam Facility for Cell Irradiation with Single Ions

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In the last 15 years, the GSI microprobe has been used for different applications like ion lithography, micromechanics or the investigation of the effects of radiation on microelectronics. With its ability to focus ions from carbon to uranium with energies between 1.4 MeV/nucleon and 11.4 MeV/nucleon into a beam spot with a diameter of 0.5 μm, it is also of interest for the irradiation of single biological cells. Compared to the existing single-cell irradiation facilities, which use mainly light ions like hydrogen or helium, the range of the LET can be extended considerably with heavy ions. As a focusing microprobe, it also offers a smaller beam spot and a better-defined LET because the particles are not scattered inside a collimator. As an additional bonus with a focused microprobe, we expect a higher throughput since the microbeam can be deflected to the position of the cells instead of having to move the cells into the beam position. Therefore, during the last 3 years, a number of new components were developed to expand the ability of the GSI microprobe to irradiate cells.

The microprobe is situated at the end of the GSI linear accelerator (UNILAC). The ions that enter the microprobe through a small object aperture are focused by a magnetic quadrupole lens to a focal diameter of about 500 nm. Deflecting magnets allow the movement of this focus perpendicular to the beam direction. A fast electrostatic beam switch in front of the entrance aperture connected to the hit detection system ensures irradiation with a predetermined number of particles. A more detailed description of the microprobe can be found in ref. (*1*). The new components for the cell irradiation project are presented below in more detail.

Vacuum Window

The biological cells need to be irradiated at atmospheric pressure in a nutrient solution. Therefore, the microbeam has to be extracted from the beam line at 10^{-7} mbar into the irradiation chamber at ambient pressure. The vacuum window used for this task needs to be very thin to reduce scattering of the ions, but it also needs to be radiation hard to withstand atmospheric pressure even after high-fluence irradiation.

In our setup, a round, 1.5-mm-diameter Si_3N_4 foil 200 nm thick will be used as the vacuum window. It has been tested with $1 \times 10^{11}/cm^2$ carbon ions (3.6 MeV/nucleon) and $6 \times 10^8/cm^2$ iron ions (4.8 MeV/nucleon) at atmospheric pressure without showing any alteration.

The calculated scattering of 42 MeV/nucleon carbon ions by this window over a distance of 200 μm is less than 500 nm according to SRIM-2003 (http://www.srim.org). Two hundred micrometers is the maximum distance we expect to have between the vacuum window and the surface of our sample in our experiments, so the accuracy of our setup will clearly be good enough to hit specific areas in single cells. This has also been confirmed by a number of scattering experiments (*2*).

Cell Chamber

In contrast to most other cell irradiation facilities, a horizontal beam is used at GSI. Therefore, a cell chamber is used to keep the cells in a nutrient solution in a vertical position. This chamber is closed on the beam entrance side with a thin foil onto which the cells are grown and on the back side with an optical window that allows the observation of the cells during irradiation. The 20-mm square chamber body, which is made of stainless steel (type 1.4301) with a 10-mm-diameter hole in its center, was designed to fit into a vertical X-Y stage. A 4-μm thin polypropylene film is glued with stearin onto the chamber body such that a dish is formed in which the cells can be cultured. Intensive cell culture

tests with this chamber have shown that the stainless steel and the stearin glue do not inhibit cell growth.

Particle Detection

The hit detection efficiency is one of the most important properties of a single-ion irradiation microbeam since the number of hits per cell is one of the major parameters in most biological experiments.

Since the ions do not pass through the 2-mm thickness of the cell chamber, a hit detector cannot be placed behind it. Furthermore, any additional detector material in front of the cell chamber would destroy the quality of the micro-focus. For these reasons we detect hits by detecting the electron clouds released from the vacuum window for every passing ion with a channeltron. To discriminate ion-generated signals from the background noise, the secondary electron yield should be as high as possible. Therefore, the Si_3N_4 foil is covered with a layer of CsI. Tests of this system showed a detection efficiency of 99.5% for carbon ions (3).

Positioning Systems

The overall targeting accuracy of a microbeam system is determined not only by the focal diameter and the scattering of the ions but also by the accuracy with which the cells and beam can be aligned relative to each other. In our setup there will be two possibilities for this alignment: either moving the cells to the actual beam focus by an X-Y stage or using the magnetic deflection system for the positioning of the beam.

For the movement of the sample, a special stage driven by d.c. motors and controlled by separate position sensors was developed. With this stage the cells can be moved at a maximum speed of about 200 μm/s in each direction and with an accuracy of about 200 nm. In contrast to that, the beam can be deflected up to about 2 mm in some milliseconds. The accuracy of the beam is then about 0.5% of the deflection; i.e., targets within 100 μm of the beam axis can be hit with a precision of about 500 nm. Therefore, in our experiments, the beam deflection method will be used to irradiate the cells in one frame while the stage is used to scan the whole sample.

Optical System

The rate at which the areas of interest in the samples can be found is another important parameter of a cell irradiation facility. To have versatility in the microscopic methods used in this task, an optical system was mounted behind the cell chamber that allows epi-illumination with UV and visible light and also differential interference contrast microscopy. Images are taken with a PixelFly 12 bit CCD camera and analyzed by a program written for the ImageProPlus software, which also coordinates the positioning and irradiation of the samples.

In the first experiments, the recognition of cell nuclei either will be done with staining with a UV-fluorescent dye like Hoechst 33258 or will be done manually.

Conclusion

With the changes described here, the GSI microprobe has the possibility to irradiate biological cells with single ions. The first experiments with living cells were undertaken in April 2003.

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Research and Development of the Single-Particle Microbeam Facility in the Institute of Plasma Physics

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Introduction

Single-particle microbeam facilities (1–3) have played a very important role in radiation biology research in recent years (4). With its ability to irradiate the target in an accurate position and to count particle numbers exactly, this kind of facility can give scientists a good method to study the mechanisms underlying the interactions between cells.

The accelerator of this facility is an electrostatic Van de Graaff, which can supply nearly 3.5 MV and H^+ (and H_2^+) ions. Particles (H^+ or H_2^+) from the electrostatic accelerator are bent 90° by the first bending magnet, focused by the quadrupoles, then bent 90° by the second bending magnet. Bent by 180°, those particles enter the microbeam room, which has a collimator, a sample stage, a microscope, an intensifier, a CCD camera, a particle detector, and a control computer. When a particle exits the collimator and enters a cell on the sample stage, the detector sends out a pulse to the computer. Then the computer sends out a pulse to the beam shutter to switch off the beam. The images of cells that were irradiated or not irradiated by the particles can be input into the computer from the image system (microscope, intensifier, CCD and A/D). After the computer processes the image, it will send out another pulse to the beam shutter to open the beam to another cell on the sample stage.

The Microbeam Experiment

The ratio of H^+/H_2^+ in this accelerator is near 1:1. To get a small-diameter spot at the end of the beam line, collimators were used. The collimator is a stainless steel chip with an aperture of either 300, 55, 30 or 10 μm diameter. To seal the vacuum, a 3.5-μm-thick vacuum film (Mylar) was used after the collimator. Because the beam line is too long, the vacuum near the collimator is around 1×10^{-2} Pa, whereas the best vacuum in the tube of the beam line is near 1.0×10^{-4} Pa. If the Mylar film is broken, the vacuum would deteriorate quickly, but 3.5-μm-thick vacuum film is adequate.

Because the beam current at the end of the beam line is so low (pA–nA), we set up two pre-collimators between a 2-mm diaphragm and the collimator. These pre-collimators are pA meters that can measure the beam currents in four directions (east, west, north and south). When the beam current of the east–west or north–south axis is nearly equal (or the beam currents can be measured in all four directions), a micrometer beam can be obtained after the collimator and the vacuum film. The H_2^+ tracks on the solid track detectors (CR-39 film) etched in the NaOH solution showed that the beam went through both the apertures (2000, 300, 55, 30 or 10 μm) and the 3.5-μm-thick vacuum film (Mylar).

After the solid track detection, the energy spectrum detector has measured the beam after the apertures and the 3.5-μm vacuum film. The H^+ energies in two experiments are near 3 MeV. The measured energy spectrum has also shown that the beam can go through the small apertures and the 3.5-μm-thick Mylar film.

Images of 10–20-μm-diameter particle spots on the CR-39 film when the distance between the collimator and the sample stage is near 10 μm (the diameter of the collimator aperture is about 10 μm) have indicated that the initial micrometer beam experiments have succeeded. The collimator, the microscope, the microscope supporter, the detecting system and the sample stage were aligned and set up accurately, so the single-particle experiment will be successful.

Future Developments

Some parameters of this microbeam facility need to be improved after the initial basic experiments. For the principal experiments, the low (10^1–

10^2 cells/h) irradiation speed is enough, but in the future the speed needs to be of the order of 1000–3000 cells/h for the cell irradiation experiments. Because a small beam spot is essential in this facility, optimizing the beam line, decreasing the diameter of the collimator, and decreasing the distance between the collimator and the sample stage will be required.

Conclusion

A micrometer-sized beam was obtained in the microbeam facility of the Institute of Plasma Physics, and the single-particle experiments are on schedule. The parameters of this facility need to be improved since the experiments and construction are still at a preliminary stage.

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Design, Characterization and Application of a Charged-Particle Microslit for Subnuclear Irradiation

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Subnuclear irradiations have been performed with a collimated microslit using the charged-particle accelerator at the Laboratory for Accelerator Beam Applications at the Massachusetts Institute of Technology, Cambridge, MA (MIT LABA). The precision that microslit irradiation provides is of key importance to radiobiological studies for a number of reasons: (1) It allows the experimenter to precisely control the amount of radiation deposited in a given cell. (2) It allows investigation of the distribution of radiosensitivity across a nucleus, permitting varying levels of radiation intensity. (3) Defined subnuclear distribution of radiation dose allows visualization of the localization of damage repair proteins and the evolution over time of the repair of DNA damage (*1*).

Protons (750 keV) were generated using the 1.5 MeV charged-particle accelerator at the MIT LABA. The microslit was constructed by laser drilling by Lenox Laser Corporation (Glen Arm, MD). It consists of a 45-μm-thick stainless steel substrate with a 1.8 μm × 1-mm slit in the center, mounted on a vacuum assembly. Normal human fibroblast cells obtained from the Radiation Oncology Department at Massachusetts General Hospital were maintained in Dulbecco's modified minimum essential medium with 4.50 μg/ml glucose, supplemented with 10% fetal bovine serum (Sigma), 10 μg/ml streptomycin and 10 μg/ml penicillin (Mediatech CellGro), and 10 m*M* Hepes buffer (Hyclone).

Cell Irradiation

Irradiation dishes were prepared using a custom-made stainless steel tray onto which a 4-μm-thick polypropylene film was affixed. After assembly and UV sterilization, the film was treated with poly-L-lysine (Sigma) to enhance cell adhesion. Exponentially growing cells were trypsinized into single-cell suspensions and transferred to the irradiation dishes such that the cells after adhesion would be 90–100% confluent. Cells were incubated for 12 h and then irradiated at room temperature (22°C). Irradiations lasted 5–30 min, depending on area irradiated and the total dose delivered. At times ranging from 10 min to 2 h after irradiation, cells were washed with 22°C 1× PBS and then fixed with 100% methanol on ice.

Beam dimensions at the midline of the irradiated cells were determined by extrapolating the aspect ratio of the slit (25:1) through the intervening distance and adding the contribution from radial straggle caused by interactions with the intervening material (1.4 μm of Mylar, a conservative air gap, 4 μm of polypropylene, and the cells themselves) as modeled by the SRIM 2003 code (*2*, SRIM-2003; http://www.srim.org). The calculated beam profile was determined to be 2.4 ± 0.18 μm and was subsequently verified by irradiation of LR115 track-etch film (http://pro.wanadoo.fr/dosirad/Notice%20LR115-A.html). Dose was delivered in 2-μm swaths over 5–10-mm lengths, such that multiple "stripes" were delivered over the area of the cell dish. The dose in the irradiated areas was approximately 300 Gy, while unirradiated areas received no dose. The dose was calculated using LR115 film to determine the proton fluence, and the SRIM 2003 code was used to determine the dose per proton.

DNA Damage Assay

To visualize DNA damage and the localization of the DNA damage response, immunocytochemical techniques were used. After fixation, cells were permeabilized for 5 min. Cells were incubated in blocking solution for 1 h and then treated with anti-MRE11 (Oncogen Research Products) or with anti-phosphorylated histone H2AX (Trevigen) and a secondary FITC-conjugated antibody (Sigma) and then counterstained with DAPI. Images were captured under epifluorescence with a charge-coupled device camera and processed using Microsoft Photo Editor.

Treatment with anti-MRE11 allowed visualization of one of the DNA repair proteins associated with the repair of DNA double-strand breaks (*3, 4*), and treatment with anti-phosphorylated histone H2AX allows for direct visualization of the DNA double-strand breaks in chromosomal DNA (*5*). Examination of the images demonstrated the ability of the microslit, in conjunction with the MIT LABA charged-particle accelerator, to generate a pattern of radiation dose over a defined subnuclear volume. In the future, this approach will be used to explore the distribution of radiosensitivity over the cell nucleus and to investigate the dose and time response of DNA damage repair proteins.

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Update on the Laser Ion Source for the Columbia University Microbeam

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A laser ion source, a proposed replacement for the ion source on the 4 MV Van de Graaff accelerator at the Columbia University Radiological Research Accelerator Facility (RARAF), offers an exciting upgrade to expand the LET range for irradiation experiments with mammalian cells. Through laser ablation, the laser ion source can produce heavy ions with high charge states from a solid target; after acceleration, these ions will have sufficient energy to irradiate cells on a thin surface at atmospheric pressure. A high-power 100 Hz pulsed Nd:YAG laser used with the laser ion source has produced aluminum ions with charge states greater than nine. Proper power management issues are important in obtaining the high charge states while protecting sensitive laser optics. We expect that the laser ion source will enable us to use ions of sufficient range from hydrogen to iron, providing a range of linear energy transfer from about 10 to 4,500 keV/μm.

Session IV

Chair: M. Hill

MRC Radiation and Genome Stability Unit, Harwell, United Kingdom

Development of Photon Microbeam Irradiation System for Radiobiology

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Evaluation of the risk of exposure to low-dose or low-dose-rate radiation has been a great concern for human societies using modern technologies. The energy of radiation, after conversion to kinetic energy of secondary charged particles, is deposited within the molecules in cellular systems. The energy deposited in the cell, or dose, is proportional to the number of particle traversals across the cell. In low-dose regions, the average number of tracks per cell becomes small, and hence the number shows a Poisson distribution. From the characteristics of the Poisson distribution, the percentage of cells that have not received any track at all is 37% when the average track number is one. At doses lower than this, the number of non-hit cells becomes greater than the number of hit cells. Observation of the total cell population without knowing which cells were hit and which were not hit would give very little information on the mechanisms of cellular responses to low-dose radiation under environmental circumstances. We need to know whether individual cells are irradiated or not to study the radiation response of the cell. Because of this requirement, microbeam irradiation systems using particle (α or proton) beams have been developed in the UK (*1*) and the U.S. (*2*). According

to recent reports, it was found that the non-hit cell situated near the irradiated cell exhibits a response to the radiation (bystander effect). This type of response can be observed only when the individual cell is identified and irradiated with the desired dose using a microbeam a few micrometers in diameter.

Considering that we are exposed to γ- or X-ray photons more often than to high-energy heavy particles in our living environment, we decided to develop a microbeam irradiation system using monochromatic synchrotron X rays to study the response to low-dose X rays.

Design of System

The proposed system is composed of three parts (*3*). The first is a Karkpatrik Baez (K-B) mirror system to focus the X-ray beam to the size of 1 μm in diameter. Then the beam is reflected 90° upward by a silicon crystal (311), which selects the energy of X rays to be 5.35 keV. The range of the photoelectrons generated with X rays of this energy is about 0.8 μm. Second is a fluorescence microscope equipped with a precise motorized stage on which the sample dish is fixed and irradiated with a focused X-ray beam from below. The third part is a fluorescence image analyzer (computer) with a sensitive CCD camera that recognizes the target cells and their positions. This computer also controls the application of the X-ray beam to the target cells, one by one, automatically. The planned throughput is 1000 cells per hour to keep the cells in good physiological condition during the irradiation process. This system is now being installed at BL-27B in the experimental hall of the 2.5 GeV electron storage ring in the Photon Factory, Institute of Materials Structure Science, High Energy Accelerator Research Organization (KEK) in Tsukuba, Japan. Experimental stations at BL-27 are situated in the biological sample preparation area, where incubators and other equipment to grow and keep mammalian cells are available. Presently, we can irradiate cells in ''Point and Irradiation'' mode.

Acknowledgments

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Microbeam Studies of Low-Dose X-Ray Bystander Effects on Epithelial Cells and Fibroblasts Using Synchrotron Radiation

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Evidence is emerging that radiation exposure can change communication between cells of the same type as well as between cells of different

cell compartments within tissues. We are using the X-ray Microprobe Beamline (10.3.1) at the Advanced Light Source (ALS) to investigate bystander effects from low doses in well-characterized human mammary epithelial cells (HMEC) and human skin fibroblasts (HSF). The discovery of ''bystander'' effects on unirradiated cells from radiation-exposed neighbors has confounded and challenged radiation researchers. It has been difficult to understand how unirradiated cells could be affected. The ALS facility is capable of producing a beam of 12.5 keV X rays with a focused spot size of 2 μm^2 and a wide range of doses and dose rates. Unlike normal X-ray sources, this beam has a very low background of both low- and high-energy X rays. In initial studies, a multilayer monochromator was used to produce a 100-μm-wide beam, 12.5 keV fan beam. A 100-μm-wide stripe of radiation was created by scanning the fan beam across the cells at a constant scanning rate. The dose rate was varied by adjusting an upstream slit that varied the width of the beam in the scanning direction. During irradiation, the dose rate was monitored with an ion chamber that was downstream of the slits and upstream of the monochromator. The X-ray dose rate was calibrated by placing a NaI photomultiplier detector (100% efficient at 12.5 keV) at the sample position and then calculating the relative counting rates of the ion chamber to this detector. The cell cultures were grown in microwell slide chambers that have four 9-mm-wide compartments separated by 1 mm. To evaluate the spatial dependence of intercellular communications, we varied the distance between dose stripes from 100 μm to 900 μm. A computer-controlled, quantitative fluorescence microscope was used to evaluate several classes of radiation-induced soluble signals, how these signals are communicated across cell compartments, and how radiation changes cell signaling both acutely and chronically. In particular, we have used immunohistochemistry to measure the induction by radiation of p21[Cip1] (CDKN1A) and phosphorylation of H2AX and p53 serine-15. Cellular responses to doses from 4 Gy down to 2 cGy were examined over a time course from 10 min to 12 h after exposure. Preliminary results indicate that there is a dose- and cell-type-dependent expression of p53 serine-15P within 10 min after exposure to a 100-μm-wide stripe of dose, with epithelial cells responding more rapidly and with greater intensity than fibroblasts. We have also quantified the number of p53-serine-15P-positive cells in the unirradiated cell populations between two stripes of dose as a measure of the bystander effect and compared these data to the appropriate controls.

Soft X-Ray Optics: Problems and Prospects

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This paper describes the difficulties inherent in optically manipulating X-ray beams and how they may be overcome. The difficulties arise since the refractive index is very close to unity for all materials, leading to small refraction angles and thus long focal lengths and poor imaging quality for conventional lenses. Additionally, absorption is high, and reflectivities at near-normal incidence are small. Hence different approaches must be used in the design of efficient optics for X rays. Suitable methods include grazing incidence reflection, the enhancement of near-normal incidence reflectivities using multilayer stacks, linear arrays of refractive lenses, and, perhaps most successfully for high-resolution applications to date, diffractive lenses.

When Röntgen discovered X rays (*1*), he tried, unsuccessfully, to detect effects due to their refraction, reflection and interference, concluding that the refractive index was less than 1.05 so that ''X rays cannot be con-

centrated by lenses''. Note that this assumed that X rays are a form of electromagnetic radiation, and initially there was some debate over this. In the following years the evidence that X rays are short-wavelength electromagnetic radiation gradually accumulated, beginning in 1899 when Haga and Wind passed X rays through a V-shaped slit and, from the narrow end, saw evidence of diffraction leading to a wavelength estimate of ~0.1 nm (*2*) (this was, of course, before quantum physics and the concept of wave-particle duality). A few years later, Barkla (*3*) showed that X rays could be polarized, like light, suggesting a similarity between X rays and other forms of electromagnetic radiation. In 1913 von Laue (*4*) realized that interatomic distances are of same order of magnitude as X-ray wavelengths, as determined by Haga and Wind, so that crystals should act as three-dimensional diffraction gratings. This diffraction was observed by Friedrich and Knipping in the same year and led to its interpretation using the well-known Bragg law.

Crystal diffraction led to the construction of X-ray spectrometers (*5*), allowing the first systematic measurements of X-ray spectra and wavelengths and crystal structures. Diffraction studies also led to the first observation of X-ray refraction, the angles of peak reflectivity for X rays incident on gypsum and sugar crystals being slightly greater than those predicted by the Bragg law. This was due to refraction at the air/crystal interface and indicated that the refractive index was slightly less than unity. It was thus suggested that X rays could be totally externally reflected at an interface, analogous to total internal reflection when visible light is incident on the surface of a swimming pool from below. Such external reflection was first observed by Compton in 1923 (*6*), and, although it cannot be total, due to absorption, this led to the first form of viable X-ray optic. However, since X-ray refractive indices for all materials are very close to unity, high reflectivities are obtained only at very small angles with respect to the surface (grazing incidence). This leads to severe aberrations—astigmatism, spherical aberration and coma—unless complicated surface shapes or multiple reflections are used. Multiple (normally two) reflections help since the second reflection may compensate for the aberrations introduced by the first. Common systems are Kirkpatrick-Baez (*7*) and Wolter (*8*) optics, the former employing successive reflections from crossed cylindrical (or spherical) surfaces and the latter using reflections from two conic sections. More recently, polycapillary (*9, 10*) and microchannel plate (*11, 12*) arrays, working by grazing incidence reflections along many small diameter channels, have been used, but performances are limited by channel tilting, curvature errors, waviness, diffraction, uncontrolled substrate bending, and misplaced channels (*13*). In addition, microchannel plates are made primarily for another purpose—X-ray detection—and are not optimized for optical performance; e.g., channel wall roughnesses are not a major concern for X-ray detection but can severely reduce the reflectivity. Such optics can also have very poor point spread functions (*14*). However, recent advances in micro- and nano-fabrication techniques mean that optimized microstructured optical arrays (MOAs) are now feasible (*15, 16*). The possibilities allowed by MOAs include controllable focal lengths and, for the first time in X-ray optics, adaptivity (*17*).

At near-normal incidence, reflectivities are very low; typically only one in 10^5–10^6 of incident X-ray photons will be reflected. However, if many reflections can be made to add in phase, then the overall reflectivity can be much higher (*18*). This is the same principle as used in crystal diffraction, but for longer-wavelength X rays, no stable crystals with appropriate interplanar spacings exist. Synthetic structures, consisting of alternating layers of different materials and known as multilayer mirrors (*19*), are, however, widely used. Recent advances in this concept use more than two materials (*20*) and optimize the layer spacing distribution to give customized reflectivity profiles as a function of angle (*21, 22*) or wavelength (*23*).

The closeness of the refractive index to unity also means that refraction angles are very small, so that single refractive lenses for X rays would have impractically long focal lengths. Linear arrays of lenses have been used for high-energy X rays (*24*), but absorption limits their use at lower energies. In their earliest implementation, these compound refractive lenses were simply lines of closely space cylindrical holes drilled into alu-

minum blocks. The walls of successive holes acted as concave lenses that gradually focused the X rays, the refractive index of aluminum being less than that of air or vacuum. The overall effect was similar to that of a cylindrical lens, i.e. a line focus of a point source. Parabolic profiles have now been implemented to reduce aberrations (*25*). Similar lenses, with similar properties, have been made using two identical saw-tooth arrays placed with the teeth facing each other (*26*). These have the advantage of variable focal length since their distance apart may be changed.

For much of the X-ray wavelength range, diffractive optics offer the best performances to date, specifically zone plates: circular diffraction gratings with radially increasing line densities. To understood how they work, first consider a linear transmission grating; some incident radiation passes straight through (the zero order), some is diffracted to one side (the positive orders), and some is diffracted to the other side (the negative orders). A circular grating with a constant period would thus form an axial line focus of a point source. For smaller periods, radiation diffraction angles are larger, and hence, if the period decreases as the radius increases, the distance to the focus can be made constant. The grating then acts as a lens in that monochromatic radiation from a point source is brought to an axial focus. This is the basis of zone plates, the focusing properties of which depend on (1) the relationship between the zone width and its radial distance from the center, (2) the number of zones, and (3) the zone heights and profiles. Combinations of multilayer mirrors or crystals with zone plates, known as Bragg-Fresnel lenses (*27*), have also been used in some cases.

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Developing a Laboratory-Based Titanium K$_\alpha$ X-Ray Microprobe

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Introduction

The original soft X-ray microprobe at the Gray Cancer Institute was designed to operate in the carbon K-shell energy range (278 eV) (*1*). Recently, the need for increased penetration in tissue and the drive to investigate the effects from low-LET radiations has prompted us to develop a higher-energy X-ray source. While the original microprobe has been successfully upgraded to operate at characteristic aluminum energies (1.5 keV), the X-ray penetration is still limited to just one or two cell layers. To overcome this, a new X-ray microprobe is being developed. As before, it is an electron bombardment source, but built in modular sections, and using a titanium target to create characteristic 4.5 keV Ti$_K$ radiation. A high-brightness, single-crystal, lanthanum hexaboride cathode (Agar, UK) is used as the electron source.

Permanent Magnetic Lens

Within the microprobe, a magnetic lens is required to focus electrons on to the target. Our current source uses a conventional electromagnetic focusing system, which has the disadvantage that it generates heat and requires water-cooling. An alternative solution is to construct an objective lens that uses a permanent magnet to provide the focusing action (*2*). Although most magnets are too weak to create the desired focusing effect,

a neodymium-iron-boron magnet does have sufficient strength to create the required force. This method does not require cooling and has a number of other benefits, including increased stability and reduced size (indeed, the entire lens can be housed within the 50-mm-diameter of a standard vacuum tube). Because different electron energies may be required, the field strength of the lens must be variable over a useful range. This can be achieved by using an adjustable magnetic slip-ring that surrounds the magnet but is located outside the vacuum for ease of access.

The lens itself was designed semi-empirically using finite element analysis software (Field Precision, Albuquerque, NM), including a consideration of non-linearity and saturation characteristics and the focusing action of the slip-ring. It will be comprised of a neodymium-iron-boron annular magnet, with a soft iron yoke and pole-piece, and is designed so that all the flux is contained within the magnetic circuit. The amount of metal used is minimized while preventing saturation and magnetic flux leakage into the bore, which would lead to the formation of parasitic magnetic lenses. Elsewhere within the system, austenitic stainless steel is used due to its very low magnetic permeability. Plotting the magnetic-field distribution, B_z along the optical axis, we find that it is only within the 2.4-mm-diameter aperture of the pole-piece that an intense field is established. Ray tracing of electron paths has allowed the theoretical optical properties of the lens to be established. Curves showing the variation of focal length with axial magnetic field were plotted for electron kinetic energies of 10, 15 and 20 keV. Since the anode position is fixed, the focal length of the lens must remain constant. The simulation can be used to estimate the required magnetic field, to obtain the correct focal length, at a range of electron energies. To obtain a focal length of 5.5 mm, the peak field must be 200, 240 and 270 mT for 10, 15 and 20 keV, respectively. Overall, our current estimates indicate that the focusing system will have a demagnification factor of 15.

X-Ray Emission

To maximize the X-ray emission, the intention is to operate at higher energies and beam currents than the existing microprobe. Using higher energies increases the efficiency of X-ray production; the efficiency of X-ray production doubles between 10 kV and 20 kV, with the total Ti_K line intensity increasing by a factor of 6. A benefit of running at energies above 10 keV is that the peak in the unwanted continuum (bremsstrahlung) spectrum is shifted away from the line emission and therefore makes a lower relative contribution in that wavelength range.

Working at high beam powers presents a problem when focusing to micrometer-sized spots, since the increased power density has the potential to cause target melting. To avoid this, we are designing a rotating anode using a ferrofluidic UHV bearing (Rigaku, Danvers, MA), and a water-cooling system for the target. The finite-element analysis calculations show that the magnetic interaction of the target assembly will not have a perturbing effect on the electron focusing system. To ensure a balanced anode, it will be precision-manufactured and then coated with a layer of titanium using electron-beam evaporation.

X-Ray Focusing Methods

Historically, the development of X-ray focusing methods has concentrated on energies below 2 keV. We are currently working on two quite different approaches with regard to the method used to focus 4.5 keV Ti_K X rays. The first approach is based on a method commonly used in X-ray microscopy and is the method used for our first-generation microprobe. The emitted X rays will be reflected at grazing incidence from a multilayer mirror. This mirror will be optimized to reflect low-energy radiation, at 4.5 keV and below, while cutting out undesirable bremsstrahlung radiation at higher energies. Low-energy bremsstrahlung is filtered out by transmission through the vacuum window. A high-efficiency tungsten zone plate (King's College, London) then focuses the radiation to a point. To achieve a sub-micrometer focused spot, a demagnification of at least five is required (assuming a <4–5-μm electron spot diameter).

Standard amplitude zone plates have a theoretical maximum diffraction efficiency of 10%, in the first order, but in practice are much less. They are typically no more than a few hundred micrometers in diameter, due to manufacturing constraints. Other types of zone plate may be more or less efficient, but all will suffer from small-collection solid angles at large demagnifications. To address this problem, we are working in collaboration with King's College London and the University of Birmingham to exploit microstructured optical arrays (MOAs). These arrays focus radiation by double reflection from the outer walls of radially increasing channel widths. They have the advantage of being achromatic, and because the innermost channel is the smallest, they can be made much larger than other optics (i.e. up to several millimeters in diameter). Simulations indicate that they will perform at least an order of magnitude better than a standard zone plate (3). We have designed a prototype MOA and jig for testing on the existing microprobe (3). This prototype will form the basis of a three-dimensional version for the new system.

Conclusions

The development has thus far centered on computer modeling and optical design. It can be seen that several novel ideas have been combined with more conventional techniques. We have recently begun to manufacture and test some of the components. The control systems and imaging facilities will be added at a later stage using an approach that will complement our existing micro-irradiation facilities.

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Session V

Chair: Y. Kobayashi

JAERI, Takasaki, Japan

Cell Imaging Techniques for Use with Microbeams

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Increasingly sensitive assays are required to measure the effects of individual particle traversals through individual and neighboring cells. There is every reason to anticipate technological improvements in the ability of particle and microfocus X-ray beam delivery systems in terms of both targeting accuracy and penetration. This will place increasing demands both on "target" imaging methods and on systems to quantify radiation effects. Many micro-irradiation studies require a high cell throughput and the minimization and/or elimination of photodamage associated with additional insults to the cells. Other studies are associated with transmission of effect to neighboring cells, and it is clear that future planned studies will require localization of the target volume in "thick" samples.

It is important to consider (1) the improvements in existing optical imaging techniques, (2) methods currently not employed but that have potential applications in microbeam biology, and (3) available methods

for imaging in thick samples. To some extent, most of the currently used imaging devices attempt to balance the disparate requirements of high throughput and accuracy through a range of engineering compromises. Several advantages are offered by the use of ''offline'' imaging stations, particularly since these can use well-established methods that require optical access to both sides of the sample, which normally is not possible in almost all sequential irradiation setups. For example, Hoffman modulation contrast illumination methods use oblique transillumination to render transparent objects visible by giving an intensity modulation to object refractive index heterogeneities. With these techniques it is possible to identify the cell nucleus by the refractive index difference at the nuclear membrane, where denser nuclear material is separated from the cytoplasm. Hoffman microscopy also has the advantage of a degree of optical sectioning. However, in some cases, accurate optical sectioning is needed and the potential of spatially modulated, structured light, illumination techniques will be presented.

Intercalating dye-based fluorescence microscopy currently is used routinely to delineate the cell nucleus, which can then be automatically identified and positioned. Other studies of cell signaling pathways require the targeting of subcellular structures such as mitochondrial-rich regions and then the revisiting of cells to monitor changes in e.g. calcium, cytochrome c, caspase levels and oxidative activity using a range of molecular markers and stains. Minimizing phototoxicity is almost always required, and non-linear excitation methods such as multi-photon microscopy could be used to advantage. Some recent developments have occurred in fluorescence steady-state imaging well below the diffraction limit, with far-field imaging apparatus. In some cases, a near-field resolution (<10 nm) is possible by exploiting time-resolved techniques in conjunction with FRET (fluorescence resonance energy transfer) methods.

Nevertheless, most routine work will inevitably be based around rather more conventional, but highly optimized, camera-based microscopy arrangements. In all methods, image processing plays a crucial role in delineating objects of interest and analyzing effects. However, such processing must be seamlessly integrated with image acquisition and sample positioning systems. By way of an example, a high-throughput arrangement, based on readily available component parts, has been developed. Although optimized for automated ''comet'' analysis, it will form the basis for the next generation of micro-irradiation and offline imaging devices under development at the Gray Cancer Institute.

Microdosimetry Calculations for Low-LET Microbeam Facilities

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The bystander effect, responses of cells not traversed by particles of a radiation field, has generated a flurry of activity in the radiation biology community. The conventional wisdom that health risks are determined by the response of individual cells to DNA damage is being challenged. Much of the current data on bystander effects was obtained in experiments with high-LET radiation, where most of the cells are bystanders and their responses are more easily observed. Even at a low fluence, radiation fields composed of low-LET particles tend to produce a uniform distribution of dose at the cellular level, which makes bystander cells more difficult to identify. Despite these difficulties, experimental results on bystander effects induced by low-LET radiation are becoming available through application of focused X-ray sources (*1*) and electron microbeams (www.microbeam.tamu.edu/elec-micro.shtml). We have applied Monte Carlo track structure simulations (*2*) to calculate microdosimetric quantities that are useful in the design of experiments with these facilities.

Low-LET Microdosimetry—Electrons

The PITS code set (*3*) was used to generate electron tracks at primary energies of 25, 30, 50, 60 and 80 keV based on the MOCA8 (*4*) set of elastic and inelastic electron-impact cross sections for water. The probability of an energy deposition event was scored for 1-μm-diameter spheres located at various lateral (*r*) and forward (*h*) penetrations. Distributions of event size were also scored and used to calculate the average amount of energy deposited in a sphere at (*r,h*) if an electron passed through it. To maximize the use of each simulated track, equivalent sites (non-overlapping spheres at the same distance from the beam axis and fixed penetration) were scored individually and subsequently combined. Scoring was carried out in short runs of 10^5 tracks so errors could be estimated for mean values of event frequency and size for at least 1 million tracks. The product of these mean values divided by the volume of the spherical site was interpreted as an ensemble average of the density of energy deposited at the center of the site by an electron of the specified primary energy.

We used weighted linear and non-linear least-squares methods to optimize parameters of analytical functions that captured the dependences of the event frequency and mean event size on *r* and *h* at each of the electron energies where datasets were generated. Weights were the reciprocal of the standard deviation of 10 independent replicates of the quantities calculated for each site. At the primary electron energies where tracks were simulated and scored, data fitting techniques, such as B-splines (*5*), can reproduce the Monte Carlo results more accurately than the analytical representations obtained by curve fitting. However, the analytical representations of event frequency and mean event size can be used at intermediate electron energies between 25 and 80 keV where Monte Carlo results are not available.

Results obtained by simulating single electrons injected into a uniform water medium can be extended to multiple electron tracks by application of binomial statistics. The probability that exactly *n* tracks deposit energy in a sphere centered at *r* and *h* when a pulse of $m > n$ electrons is injected into the medium is given by

$$P(n) = \frac{m!}{n!(m-n)!} F^n (1-F)^{m-n}, \tag{1}$$

where *F* is the probability that a single track deposits energy in the site. The event frequency in a sphere centered at *r* and *h* is

$$P_{\text{hit}} = \sum_{n=1}^{m} P(n). \tag{2}$$

The mean event size is

$$\langle Z_m \rangle = \langle Z_1 \rangle \sum_{n=1}^{m} n P(n) / P_{\text{hit}}, \tag{3}$$

where $\langle Z_1 \rangle$ is the mean event size for single electrons.

If the probability that a single track deposits energy in a site is near unity, then P_{hit} is also near unity, and the main effect of multiple electrons is to increase the mean event size in the site. If the probability that single tracks deposit energy in a site is low, then the main effect of multiple electrons is to increase probability that a single track passes through the sphere; hence the mean event size is not greatly affected.

Low-LET Microdosimetry—Soft X Rays

Capabilities for soft X-ray dosimetry calculations have been extended by introduction of simulated photon focusing and also bremsstrahlung production as a potential background radiation; the implementation of each feature is being tested and verified. Computation of distributions of event size at (*r,h*) has been resumed after introduction of the liquid-water model for low-energy electron interactions (see *Liquid Model*). As described above for electrons, the probability of an energy deposition event is scored in spheres located at various lateral (*r*) and forward (*h*) penetrations, but for a range of smaller sphere diameters (down to 2 nm), since the energies of the photoelectrons are very small (278 and 1487 eV). Distributions of event size were also scored and used to calculate the average amount of energy deposited in a sphere at (*r,h*) if an electron passed through it. The basic features of the mean values are unchanged

by introduction of liquid water cross sections; preliminary indications are that the simulated track structure of the photoelectrons is more compact, which makes the microscopic energy densities greater than for previous simulations using vapor cross sections.

Software Tools for Complex Targets

Our characterization of energy deposition by 25 to 80 keV electrons in terms of the spatial dependence of event frequency and mean event size in micrometer-scale targets enables rapid calculation of the mean dose delivered to particular cells in complex geometrical arrangements. The cells of interest might be part of a multicellular target, such as an explant (*6*) or an acinus (*7*), where the difference between doses to cells targeted by the microbeam and their bystanders is an important aspect of the experimental design. In such experiments, factors like beam energy, beam size, window thickness, and depth into the sample have a large impact on the amount of energy delivered to particular cells. Even though this approach ignores statistical fluctuations in dose, it may be useful when the volumes of interest are large enough to diminish the importance of statistical fluctuations and/or when the number of experimental variables to be considered makes the Monte Carlo method impractical.

To facilitate this approach, we are developing computational tools including an interactive graphical user interface. The interface presents three sets of axes showing contour plots in the (r,h) plane of the logarithm of the event frequency, the mean specific energy, and the logarithm of the density of energy deposited. These plots are controlled by user's choice of electron energy, maximum values of r and h, number of contours, and mesh resolution. An ellipse, which represents a cellular cross section, is superimposed on each contour plot. The size and shape of the cellular cross section are varied by interactively dragging its boundary and it is moved by dragging its center. During these operations, readouts of the diameter, thickness and volume of the cell are updated continuously along with the total energy deposited in the volume and the dose in centigrays. The number of electrons in the pulse and the beam radius can also be controlled by the user.

Liquid Model for Low-Energy Electron Transport

Low-LET microdosimetry calculations have been extended to condensed phase by adding new algorithms to the PITS code suite. The Dingfelder-GSF model for liquid-water cross sections is used to construct probability tables for inelastic interactions (*8*).

For elastic scattering cross sections, experimental data are used for electron energies less than 1 keV and theoretical calculations are used for energies greater than 1 keV. A self-consistent set of experimental differential elastic scattering cross sections for electrons in water vapor (*9, 10*) are interpolated to provide probability tables for angular scattering at electron energies less than 1 keV; for energies greater than 1 keV, atomic additivity is assumed and cross sections for hydrogen and oxygen are extracted from the NIST Standard Reference Database 64 (http://www.nist.gov/srd/nist64.htm).

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Sensitivity of Electron Transport Calculations to Elastic and Inelastic Scattering Cross Sections

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Monte Carlo charged-particle track structure codes have become an important tool in radiobiology to estimate the initial spatial patterns of energy deposition. These spatial densities of energy deposition and the concomitant patterns of molecular damage are closely associated with the degree of biological response anticipated from exposure to different types of radiation. Of particular significance to radiobiology are the effects of electron slowing and production of secondary electron-induced interaction clusters in the vicinity of DNA and DNA-related structures in mammalian cells. The extent of multiple damages, and their relative locations and types, can lead to wide variations in the severity of the damage, i.e. in the capability of the cell to repair the damage, the fidelity by which it is repaired, and the ultimate viability of the cell. With regard to the repair of radiation-induced DNA damage, clustered interactions that occur within a few ångströms to several nanometers can be particularly important.

Monte Carlo charged-particle track structure models have traditionally relied on collision theory appropriate to "fast" charged-particle interactions and to experimental guidance based on gas-phase data. These data are particularly unsatisfying when the resulting models are used to predict event histories for low-energy electrons in tissue. Traditionally Monte Carlo track structure simulation codes have ceased to follow secondary electrons with energies less than about 10 eV because of uncertainties in low-energy electron cross sections in "tissue", and because of the excessive computer time needed to follow these interactions, which have been characterized as predominately elastic collisions. As we seek greater information on the spatial damage to DNA structures, however, the fate of these low-energy electrons becomes central to our understanding.

Two new sources of data make it possible to re-evaluate the applicability of assumptions made in the modeling of low-energy electron transport. First, there are new data for the elastic and inelastic scattering of low-energy electrons in condensed-phase material from the work of Sanche and his coworkers (*1*) at the University of Sherbrooke; second, new data are becoming available for the emission of electrons from thin films after charged-particle transport. The former provide new sources of cross sections for the models, and the latter provides an experimental test of the results of the models.

We have been working with the "liquid"-phase Monte Carlo charged-particle track simulation PARTRAC developed at GSF to incorporate new

low-energy electron cross sections and to extend the applicability of the code to electrons with energies as low as 1 eV. By comparing the Monte Carlo results using different electron cross sections, and by comparison of results with the gas-phase version of the Monte Carlo code PITS developed at WSU, we are beginning to explore the sensitivity of the electron transport process with respect to the applied interaction cross sections.

A comparison of PARTRAC calculations of the yield of electrons ejected from a thin carbon foil by fast MeV protons confirms the reliability of the code to describe the transport of electrons with energies larger than about 100 eV. This is to be expected because the cross sections used in the code are considered reliable for ''high''-energy electron collisions, and these cross sections are relatively independent of molecular target structure. At lower electron energies, the calculated yields increase to nearly a factor of 10 larger than the measured yields from carbon foils. Unfortunately only preliminary measurements exist for water (ice) foils, and these yields appear to fall about midway between the calculation for water and the measured carbon yields.

To explore the sensitivity of Monte Carlo results to the input data used, we first compared the results of PARTRAC (liquid) and PITS (vapor version). Again, for electron energies greater than 100 eV, the two calculations are in good agreement. At lower energies the yields from the liquid code are nearly a factor of two larger than the vapor results, reflecting the reduced ionization potential, i.e. larger ionization yields, in liquid. The rapid increase in the differential yield of electrons as the ejected electron energy decreases is about the same in vapor as for liquid, and both are at odds with the more gradual increase observed in the experiments with a carbon foil.

To investigate the low-energy portion of the transport spectrum with the PARTRAC code, it was necessary to modify the code to follow electrons below 10 eV; electrons are produced with energies below 10 eV in the slowing of faster electrons, but they were simply not transported further. Our first attempt to transport the low-energy electrons was to randomize the direction of electrons produced with energies less than 10 eV and to use a mean free path comparable to a 10 eV electron to determine if they could exit the foil. This resulted in a smooth transition in the spectrum of ejected electrons as the ejected electron energy falls below 10 eV. Such a model is unsatisfactory, of course, since it does not implicitly include the elastic and inelastic scattering. To explore more rigorously the transport of low-energy electrons, we next incorporated the inelastic cross sections measured for water-ice by Sanche and colleagues at the University of Sherbrooke and extrapolated the elastic cross sections previously included in PARTRAC to the lower energies. This resulted in an ejected electron spectrum in near agreement with the previous approximation based simply on a scaled mean free path. We are currently exploring models of the elastic scattering cross sections using the data of Sanche and colleagues as a guide.

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Development and Testing of Control Software for a Charged-Particle Microbeam

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Introduction

Charged-particle microbeams are unique radiological tools that provide the capability of precisely controlling the number of particles traversing individual cells. To achieve this level of control, the various components and subsystems that compose the microbeam must be coordinated and controlled by software that acts as the interface between the physical hardware and the user. Such control software is currently being designed and tested for the charged-particle microbeam at the Laboratory for Accelerator Beam Applications (LABA) at the Massachusetts Institute of Technology.

Overview of Control Software

The control software is responsible for controlling the entire functioning of the LABA microbeam including accelerator start-up and shutdown and manipulation of all beam-line components including the subsystems of the endstation devoted to cellular irradiation. Written in a proprietary language (Pyramid Technical Consultants, Waltham, MA) similar to C++ and running under the Windows NT operating system, the control software provides an interface within which parameters for the hardware can be both set and monitored routinely by the user. In addition, the software allows the user to input parameters necessary for specification of a particular cell irradiation experiment. A third function of the software is automated protection of the accelerator in the event of unexpected and unsafe increases in pressure or terminal voltage.

Overall control of the various subsystems is broken into logical groups, with common functionality being grouped into distinct graphical user interfaces. Navigating though this interface allows the user to constantly monitor the status of the accelerator, vacuum systems and magnets during all phases of the experiment. A distinct screen on the computer display is dedicated to each subsystem of the control software. For instance, the accelerator operation interface permits manipulation of several hardware elements within the high-voltage terminal to power up the accelerator, adjust particle energy, and vary beam current. A screen devoted to the vacuum system displays pressures at various locations within the system and allows the user to change the status of vacuum pumps and gate valves. Another interface is used to set parameters associated with all steering and deflection plates as well as focusing and bending magnets. Accessing each interface is possible from any other interface by a single mouse click. This multi-page approach allows the user to interact with a simplistic interface regardless of the complexity of the overall control system. Data important for the overall integrity of the system, such as pressures in the accelerator tank, the beam line and the roughing line, are displayed on all pages so that these can be monitored continually by the operator.

Lying underneath this graphical interface is a high level of automation and a network of safety interlocks, which are implemented to ensure both ease of use and the safety of components within the high-voltage terminal. This automation is responsible for tasks such as tuning the bending magnet current to the energy of the charged particles so that it produces the exact magnetic field required to direct the horizontal particle beam vertically through the beam collimator. Incorporation of automated interlocks also supplies a significant level of protection for the accelerator since user response times are not rapid enough to prevent catastrophic arcing due to increased pressures in the accelerator tank. If pressures in the accelerator exceed a given value, safety interlocks within the control software immediately respond by setting the terminal voltage to 0, thus avoiding an arc that could seriously damage or destroy the digital electronics housed within the high-voltage terminal. Similarly, critical gate valves in the beam line cannot be opened unless required settings for the vacuum pumps meet specific conditions.

Cell Identification

Cells to be irradiated are plated on specially constructed dishes comprised of thin polypropylene affixed to a stainless steel ring. After co-incubation with a fluorescent dye, the cell dish is attached to a 2D stage and subsequently exposed to a fluorescent light source. A CCD camera observes the magnified fluorescent images of the cell dish, and a Matrox Meteor2 graphics board located in a Dell Optiplex Workstation captures

the resulting video signal. The captured image is displayed on the computer screen and, using thresholding techniques, cells (and potentially other fluorescing objects) are identified. For each identified object, several parameters (pixel count, elongation, centroid position, etc.) are logged, and averages of these various parameters are constantly maintained so that the routine can more accurately identify what is a cell and what is debris or potentially the overlap of two or more cells. If the software identifies an object that deviates substantially from these averages, then the software requests that the user manually locate the centers of these abnormal objects using the mouse pointer. An automated method of subdividing overlapping cells into individual cells is currently under development. Once all objects have been located, the 2D stage moves the cell dish to the next position in preparation for another image acquisition. Successive image captures are intentionally overlapped slightly to ensure that subsequent concatenation within the control software does not miss or double count a cell that might be positioned at the edge of a given image. This routine is repeated until all cells on the cell dish have been located.

Given the level of automation in the cell-finding routine, a built-in method of evaluating this routine's ability to accurately identify cells on the dish was developed. Using this method, the user can test the accuracy of the cell-finding routine against his/her own ability to locate cells on a given captured image, ensuring that each cell has been identified and also that no fluorescing debris is mistakenly labeled as a cell. This test can be repeated periodically for different users, cell types, fluorescent dyes, etc. In the future, more automation and testing of this type will be implemented throughout the control software. Once fully implemented, the software will present the user with a variety of experimental capabilities such as irradiating a predetermined percentage of all cells found on the dish or dividing the dish into a user-defined number of regions and irradiating only the cells within certain regions.

Session VI

Chair: E. J. Hall

Center for Radiological Research, New York, New York

Cellular and Subnuclear Response of CDKN1A (p21) after Low-Fluence Heavy-Ion Irradiation

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Over the past few years several studies using microbeams or low-fluence radiation have shown that bystander radiation responses are induced in cells that have not had energy deposited in their nuclei (1). In view of the potential impact of such low-fluence effects on space radiation risk assessment and on healthy tissue effects related to heavy-ion radiotherapy, there is also a need to study bystander responses for heavier particles. A heavy-ion microbeam probe is presently in use and under further development with the objective of cell irradiation at GSI, Darmstadt (www.gsi.de/annrep2001). The first single-cell irradiations at GSI will focus on the influence of radiation quality on bystander effects. In preparatory experiments, we used broad-field low-fluence ion irradiation and retrospective track etching as an alternative technique to determine the actual sites of heavy-ion traversal (2). This method is applied to investigate the response of cellular proteins involved in DNA damage-induced signaling and repair pathways after ion irradiation. The present study reports results on the induction and subnuclear relocalization (i.e. focus formation) of the cell cycle-related cyclin-dependent kinase inhibitor CDKN1A (formerly known as p21), a known participant in the response of cells to radiation (reviewed in ref. 3).

Experimental Methods

For these studies, normal human fibroblasts (AG1522C or GM5758, Coriell) were grown to confluence under standard conditions (95% air/ 5% CO_2, 37°C) in Eagle's MEM (Bio-Whittaker) with 20% fetal calf serum (Biochrom). For the subnuclear analysis of CDKN1A after irradiation, immunocytochemical staining of the protein using specific primary and fluorescence-labeled secondary antibody was applied, and a confocal laser scanning microscope (Leica TCS) was used for imaging, as described previously (4). To determine the exact sites of ion traversal, the cells were grown on precoated CR-39 plates that had been preirradiated with 10 MeV/nucleon carbon ions (UNILAC, GSI) to produce reference tracks (2). After exposure to ion radiation, CDKN1A immunostaining, and imaging as described above, the plates were again etched for track detection, and images and tracks were matched as reported elsewhere (2).

To measure the level of CDKN1A protein induction after irradiation, both immunofluoresence staining of the protein, as described above, and Western blotting were applied. For the latter technique, confluent AG1522C cells were harvested by trypsinization and cell extracts were prepared in RIPA buffer at several times after irradiation. Equal amounts of protein (15 μg) were separated by SDS-PAGE (12% gels) and transferred to a PVDF membrane. Membranes were probed with specific anti-CDKN1A antibody (Transduction Lab.) and anti-α-tubulin (Sigma) as a loading reference. Proteins were detected using a secondary HRP-conjugated antibody (ECL plus, Amersham) and film exposure (Hyperfilm ECL, Amersham).

Subnuclear Analysis of the CDKN1A Response after Low-Fluence Particle Irradiation

The subnuclear pattern of the CDKN1A immunofluorescence signal was detected in normal human fibroblasts after irradiation with 1.2×10^4 P cm^{-2} titanium ions (8 MeV/nucleon, LET 2100 keV/μm) or 0.5 and 1 $\times 10^4$ P cm^{-2} zinc ions (4.9 MeV/nucleon, LET 4000 keV/μm) at several times postirradiation. These fluences correspond to a mean of 1 and 2% of cells hit, respectively. Immunostaining revealed few single cells showing the formation of one CDKN1A focus immediately after irradiation and at all the times analyzed. Retrospective track etching allowed us to precisely correlate all the detected foci with actual ion traversals, with some displacement observed only at 3 h postirradiation due to the movement of cells. Clearly, foci were not induced in the nuclei of the neighboring fibroblasts.

In addition, the overall accumulation of CDKN1A was assessed in nuclei in proximity to the hit cells. The observation of 12–16 image fields (each containing on the average 30–35 cells) showed no evidence of the appearance of clusters of cells with a radiation-induced enhanced level of CDKN1A.

The images obtained (360–560 cells per time) were used to measure the integral nuclear CDKN1A fluorescence normalized to the fluorescent DNA counterstaining signal. The analysis of the integral levels of CDKN1A after low-fluence irradiation (a mean of 1–2% cells hit) with the titanium or zinc ions (at different times after irradiation) indicates a slight enhancement of the total amount of protein after 2 or 3 h. However, when on average every cell nucleus is hit at least once, the accumulation level of CDKN1A in these cells is also relatively low, as determined by Western blot analysis (see below). This makes it clear that the bystander response after heavy-ion traversal is difficult to assess in this system.

Induction of CDKN1A after Low-Fluence Particle Irradiation

The accumulation of CDKN1A in the cell population was also investigated using the Western blot technique after exposure of human diploid fibroblasts to 10 MeV/nucleon carbon ions (LET 150 keV/μm). Cell extracts were prepared for analysis at 0, 3, 6 and 24 h after irradiation. When AG1522C fibroblasts were exposed to a fluence where on average all cells were hit at least once, the maximum increase in the level of CDKN1A was around 2.5-fold. At a lower fluence (mean of 3% of the

cells hit), a slightly enhanced amount of CDKN1A protein was also observed at the later times. However, due to the limited maximum increase expected and the uncertainties inherent in the detection method, a clear conclusion could not be drawn. Expecting differences in the induction of CDKN1A in different cell lines, cells of the skin fibroblast cell line GM5758 were used for further analysis. For these cells, preliminary results indicated a higher level of CDKN1A induction at 24 h compared to the AG1522C fibroblasts. Also at 24 h after irradiation, the first experiments showed an increased amount of CDKN1A protein after exposure to low-fluence carbon ions (mean of 3% of cells hit). The protein level was comparable to that observed when all the cells were traversed by carbon ions. These results, although not conclusive, would be in line with a heavy-ion-induced bystander response.

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Synergy between Alpha Particles and Chemotherapeutic Agents: Effects in Cells Hit Directly and in Bystander Cells

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Introduction

A major challenge to oncologists is the treatment of metastatic tumors that are too small to identify by any conventional imaging modality. The attachment of radioisotopes to tumor-specific monoclonal antibodies (radioimmunotherapy) offers the potential for exquisite targeting of radionuclides to microscopic metastatic sites throughout the body. To limit the radiation dose to normal tissues, a short-range emitter is preferred. The high density of ionizations along the α-particle track (high LET) makes α particles much more effective for cell killing than photons or β particles. However, the short range of the α particles, the limited penetration of the antibody into tumor tissue, and potential non-uniform binding to the tumor site make it difficult to deliver a lethal dose to all cells or to cells at depth. This creates two populations of cells: those directly hit and bystanders. Because of these limitations, radioimmunotherapy has been most successful for targeting the individual malignant cells of leukemia, not in solid tumor metastases, where it is most needed. New strategies that can increase the extent and uniformity of cell killing (in both populations of cells: hit and bystander) during α-particle-based radioimmunotherapy could significantly improve the effectiveness of this treatment against early metastatic disease.

The reports of bystander effects to date have been almost exclusively *in vitro* studies and almost exclusively in normal cells, not tumor cells. Bystander effects have been reported for both low-LET X rays and high-LET α particles. Is there a significant bystander effect *in vivo* in solid tumors? There has been only one recent report that addresses this question (*1*). Tumor cells were prelabeled with ^{125}IdUrd, then mixed with unlabeled cells in various ratios and injected into mice to assess tumor growth rates. The inhibitory effects of the DNA-incorporated isotope on tumor growth

were greater than predicted by direct killing of the labeled cells, or the "crossfire" dose to unlabeled cells from ^{125}I in neighboring cells. This was interpreted as direct evidence for an *in vivo* bystander effect for cell killing in a solid tumor. ^{125}I incorporated into DNA produces cell killing effects that are very similar to those of high-LET radiation.

Combined-modality therapy, where radiation and chemotherapy are given simultaneously, uses only a low, radiation-sensitizing dose of chemotherapy that may not have a cell killing effect by itself. Combined-modality therapy has the potential to increase tumor cell killing without increasing normal tissue toxicity because therapeutic synergy may not require full chemotherapy doses.

We present preliminary results on the development of a model system to evaluate whether combined-modality treatment can improve the effectiveness of radioimmunotherapy. Two chemotherapeutic agents with radically different mechanisms of action are being investigated for synergistic effects with α particles.

Paclitaxel (Taxol) overstabilizes microtubules and interferes with mitotic spindle function, resulting in cell cycle arrest at the G_2/M stage, the most radiosensitive stage of the cell cycle. Paclitaxel has shown synergistic effects in combined-modality therapy when administered with the β-article emitter ^{90}Y both *in vitro* and *in vivo* (*2, 3*).

Oxaliplatin is a third-generation platinum compound that reacts with nucleophiles on the DNA bases adenine or guanine to form bulky intrastrand crosslinks that are difficult for cells to repair and can interfere with DNA replication. Oxaliplatin has demonstrated radiosensitizing properties for X rays *in vitro* and *in vivo* (*4*). There have been no reports of treatments combining α particles with either of these drugs. The objective of this proof-of-principle study is to systematically evaluate the ability of combined-modality therapy to increase the extent and the uniformity of the cell killing by α particles both in directly irradiated cells and in the bystander cells.

Results

A human prostate tumor cell line (DU-145) is being used for these studies. An α-particle cell irradiation system has been developed that consists of planar α-particle sources together with custom-made cell culture dishes with replaceable Mylar bottoms. The α-particle sources consist of americium-241 (as the powdered oxide) mixed with gold and rolled into a foil (fabricated by NRD, LLC, Grand Island, NY). The active surface of the foil is coated with 1 μm of gold, making this a sealed source. Five foils were purchased that span five orders of magnitude in activity. ^{241}Am ($t_{1/2}$ = 458 years) emits 5.48 MeV α particles, which are similar to the energies used in radioimmunotherapy (e.g. ^{211}At, 5.87 MeV; ^{212}Bi, 6.05 MeV). A holder has been fabricated to position the dishes above the α-particle source.

A Passivated Implanted Planar Silicon (PIPS) detector (Canberra Industries, Meriden, CT) was used to measure the energy spectrum of the ^{241}Am α particles inside the cell culture dish with 1.4-μm-thick Mylar in place, representing a direct measurement of the α-particle energy spectrum delivered to the cells. The α-particle energy spectrum at the cell position shows a maximum at 4.2 MeV. The residual range in water of 4.2 MeV α particles is approximately 30 μm (*5*), more than enough to completely penetrate cells growing in a monolayer on the Mylar surface. The energy spectrum was converted to an LET spectrum using the tables available in the SRIM-2000 software (SRIM-2003; http://www.srim.org). The LET spectrum for the ^{241}Am α particles after 1.4 μm of Mylar shows a maximum at 100 keV/μm and an exponentially decreasing tail that extends out to 220 keV/μm. CR-39 plastic track detectors (Track Analysis Systems Ltd., Bristol, UK), cut into 3.5-cm circles to fit inside the cell culture dishes and sit directly on top of the Mylar film, were used for an independent measurement of the α-particle flux. The data are in excellent agreement with those obtained with the PIPS detector. The dose rate to the cells on the Mylar surface was calculated from the measured track density and the average LET. Dose rates to cells on the Mylar membrane range from 0.0013 Gy/min to 13 Gy/min for the various foils.

A series of plastic masks have been designed and fabricated that can

be interposed between the α-particle source and the cells to partially block the α particles. The masks have a radially symmetrical array of circular openings. The diameter of the circular openings is varied, but in all cases the total open area of the mask is 50% of the entire area. This approach allows us to irradiate 50% of the cells in a series of dishes but change the spatial relationship between the irradiated cells and the bystanders. This approach creates a two-dimensional model system for micrometastatic tumors of various sizes where the shielded areas represent the deeper portions of a tumor beyond the range of surface-bound α particles. Even though the open area remains constant at 50%, changing the size of the mask changes the proportion of cells close to the ''edge'' between the open and shielded zones: *These are the bystander cells.* After irradiation, the cells can either be removed from the Mylar and analyzed in bulk (survival, DNA damage, apoptosis) or be stained with fluorescent probes and scored for these end points while still attached to the Mylar membrane, thus preserving the spatial orientation of the mask irradiation and allowing the simultaneous scoring of the two populations of cells: those directly irradiated and the bystanders. A number of parameters can be controlled and varied systematically: dose rate, total dose, the distance between the irradiated cells and those cells not irradiated (i.e. the mesh size of the mask), cell density, oxygen levels. Dose–response curves for DU-145 human prostate carcinoma cells have been generated for 250 kVp X rays and the ^{241}Am α particles and will serve as a reference for the combined-modality therapy and the mask irradiations. Preliminary results with a low concentration of either paclitaxel or oxaliplatin each show a small but significant synergistic effect with the ^{241}Am α particles. Combined-modality therapy irradiations with the masks in place are under way.

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Single-Cell Gene Expression and Microbeam Irradiation: The Concept of the Average Cell

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The expression of genes in terms of a cell population in general comes from samples involving millions of cells, as for example with microarray technologies. From this mixture an attempt is made to infer the state of an average cell in the population. This is rather an averaged cell. The variation among the members of the population is not known. The charged-particle microbeam enables delivery of one or more α particles at an LET of ~90 keV/μm to selected sites of selected cells. Some cells can be hit while others are bystanders. Since the single cell is known to

be the target, the responses of individual normal human fibroblasts (both hit and non-hit) have been determined by single-cell RT-PCR, for mRNA expression, and by quantitative immunofluorescence, for protein expression. This approach permits a relatively precise measurement of single-cell transcriptional states and allows for an assessment of intercell variability. In control cells, in cells known to be hit, and in non-hit bystander cells that show responses greater than those of control cells, considerable cell−cell variability is manifest. This variability is certainly not compatible with expectations of a highly ordered consistent expression in control cells or in cells responding to precise direct or less precise indirect insults. It is possible that the cellular response to these conditions does not need tight control and that the tolerance for variation is high. Such a response would imply that monitoring expression of a gene(s) at a particular time could not lead to prediction of a given phenotype. The interrogation of individual cells along with precise delivery of insult holds promise for a better understanding of the processes that lead to relatively uniform cell population responses.

Bystander-Mediated Genomic Instability in Human Lymphocytes after Single Alpha-Particle Irradiation

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The progeny of normal cells exposed to ionizing radiation exhibit delayed responses, including a high incidence of *de novo* chromosome aberrations, gene mutation and an enhanced death rate. These delayed responses, most effectively demonstrated as non-clonal mutations/aberrations in the clonal descendants of irradiated cells, may be explained as manifestations of transmissible genomic instability (*1*). Most of these heritable effects occur at a much higher frequency than would be expected from Poisson statistics of particle hits to surviving cell nuclei, suggesting that cells not traversed by radiation may be at risk. Recent studies have suggested a link between instability and bystander responses in some systems (*2*). We have previously developed techniques for the microbeam irradiation of G_0 human T lymphocytes from normal healthy donors with exact numbers of ^{3}He particles of LET 105 keV/μm (*3*). When a monolayer of T lymphocytes was irradiated under conditions where every cell in the culture was traversed by a ^{3}He ion, genomic instability was observed in 25% of the surviving cells. A high ratio of chromatid to chromosomal aberrations was observed, consistent with late-arising, *de novo* aberrations, and typifying the instability phenotype (*3*).

In the current studies, we measured the bystander response in these cells in situations where not all the cells are irradiated. Reducing the fraction of cells targeted by a single ^{3}He ion from 100% to 20% had no effect on the observed induction of instability, suggesting that bystander responses play an important role. Second, we tested the hypothesis that cytokine signaling is involved in the induction of the instability phenotype. Preliminary results suggest that cytokines present within the irradiated culture/cells/medium play an important role in the induction of the instability phenotype in the progeny of these cells.

Materials and Methods

Separated human peripheral blood lymphocytes were attached to the base of specially designed microbeam-irradiated dishes (*3*) and irradiated in G_0 with ^{3}He particles from a microbeam source (a surrogate for α

particles) under different conditions. Control dishes were also placed on the microbeam, and each of the cells present was found and revisited without particle delivery. To investigate bystander-mediated chromosomal instability, only a fraction of the cells were targeted by a single ^{3}He ion (i.e. 50% of cells targeted, 20% cells targeted, or a single cell targeted).

The relationship between cytokine signaling and chromosomal instability was investigated in the presence or absence of antibodies against TNFA during radiation. From irradiated and control cultures, T-lymphocyte cultures were established and clonogenic and non-clonogenic assays were used to assess the delayed expression of chromosomal instability in the progeny of the surviving cells.

Results and Discussion

The instability phenotype may be induced effectively by the passage of a single densely ionizing particle, similar to an α particle from radon. Our previous work (3) provided evidence of chromosomal instability in human lymphocytes when every cell is targeted with a single helium ion (i.e. 100% irradiation). Using the same experimental procedures, similar levels of instability were demonstrated when 50% or 20% of cells were irradiated. For these variant conditions, we observed a significant increase in the induction of chromosomal instability above control levels, which appeared to be independent of the percentage of irradiated cells ($P = 0.0094$). In the case of a single-cell hit, however, there was a small increase in chromosome instability above controls. These results confirm that bystander mechanisms contribute significantly to the induction of chromosome instability by high-LET radiation in human lymphocytes. There is presently considerable debate concerning the contribution of gap junction communication (4) and medium-borne factors (5) to bystander-mediated genomic instability. The current studies are limited to the supposition that medium-borne factors are culpable, since the peripheral blood lymphocytes used originally have a loose histological structure.

TNFA is important in immune system modulation, radiation response, and cancer progression (6). The relevance of TNFA was monitored by blocking TNFA activity in irradiated T lymphocytes. When TNFA was blocked, the induction of radiation-induced chromosome instability was reduced substantially to near the background level.

Chromosome instability in cells treated with TNFA protein (as a positive control) was slightly increased above control values, implying that other cytokines may also be involved. Thus it is likely that a medium-borne factor similar to that described in refs. (7, 8) may also induce long-term chromosomal instability.

Conclusions

Long-term chromosomal instability in human lymphocytes was induced in a significantly greater portion of the population than would be predicted by the percentage of the population traversed by a high-LET ^{3}He particle, including doses low enough that only a single cell in the population was targeted. The induction of the instability phenotype was substantially reduced in bystander cells when antibodies against TNFA were introduced into the medium, suggesting that cytokines play a significant role in the initiation of instability. In summary, the current results suggest that bystander mechanisms are powerful mediators of the delayed instability phenotype and that medium-borne factors, such as cytokines, play a significant role in the induction of the instability phenotype.

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Effect of a Single Argon-Ion Hit on Cultured Mammalian Cells

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The microbeam facilities at Columbia University (1) and Gray Cancer Institute (2–4) have been used to analyze the mechanisms underlying novel cellular responses such as the so-called bystander effect (5) and have provided information on new aspects of radiation biology. This phenomenon involving cell-to-cell communication overturns the classical radiation biology dogma; i.e, normally, to analyze the biological response to radiation, living cells are directly irradiated and the responses observed.

For high-LET particles of relevance to cosmic radiation and also used for cancer therapy, less has been reported about their biological effects at low fluences. Besides, since the energy transfer of a single hit with a high-LET heavy ion is higher than that of photons and protons, it is expected that the participation of bystander effects in cell killing will be relatively large, especially in low-dose radiation fields.

Thus we have established a method for irradiating single cells with the heavy-ion microbeam apparatus installed on TIARA, JAERI-Takasaki (6). With this method, we irradiated cultured mammalian cells in air with a single or precise numbers of ^{40}Ar heavy ions (11.5 MeV/nucleon) with a spatial resolution of a few micrometers.

Preparation of Sample Holder

We used a CR-39 ion track detector as the base of the sample holder. The CR-39 is 100 μm thick, so the irradiated ion can easily penetrate this layer. A postitional mark for cell inoculation was written with a needle; then a polycarbonate ring frame was bonded on the center of the CR-39 to shape the holder into a culture dish. The holder was then washed thoroughly to remove endotoxic materials and was finally sterilized with 2 kGy γ rays.

Cell Inoculation and Irradiation

We used CHO-K1 cells for our studies. The cells were kept in exponential growth and were inoculated at the place marked on the sample holder. At one time, 50–100 cells were inoculated into a 5 × 5-mm area. After 2–6 h incubation at 37°C, the individual cells were irradiated.

Positional data for the individual cells attached on the CR-39 dish were obtained with an offline microscope before irradiation. To detect the cell position automatically under a fluorescence microscope, we used a fluorescent dye (CellTracker Orange, Molecular Probe) that uses green light for excitation. Thereafter, the targeting and irradiation of the cells were performed semi-automatically at the online microscope of the microbeam apparatus according to the positional data obtained. Since our microbeam system requires that the irradiated ion penetrate the whole target sample including the cell and the CR-39 for counting and controlling the numbers of irradiated particles, the medium was removed and the cells were covered with a thin Kapton film to prevent desiccation.

Immediately after irradiation, the positions and the numbers of ion tracks that had traversed the cells were detected from the etching of the CR-39. To detect ion tracks at 37°C, we employed an etching solution containing ethanol and potassium hydrate (7). After 15 min incubation with the etching solution, the track of an argon ion was detected clearly under an optical microscope. After the etching procedure, we took photographs of both the cell and the etched pit using a digital camera, then immediately overlaid these two images in the computer software to determine where the ion actually hit the cell. Then the growth of the individual cells was observed up to 60 h after irradiation.

Effect of Heavy-Ion Microbeam Irradiation

In the control dishes, where none of the cells were irradiated, colonies of up to 20 cells grew. In contrast, the cells in the irradiated dish that were not hit by an ion showed slightly limited growth. This limited growth might be a bystander effect produced by cells in the same dish that were hit by heavy ions.

The cells irradiated with more than one argon ion through the nucleus showed complete growth inhibition. It has been reported previously that single hits by protons and α particles have only a limited effect on the survival of the irradiated cells (8, 9). In addition, our preliminary data indicated that irradiation of CHO-K1 cell nuclei with single neon ions did not induce an observable effect on cell growth. These results suggest that the effects of an ion hit increase with increasing LET. Nevertheless, when we irradiated CHO-K1 cells with a broad ion beam, the RBE of the argon ions was lower than that of the neon ions in terms of cell killing. This conflicting result could arise from the Poisson spatial distribution of low-fluence ion-beam radiation. Thus we conclude that accurate information on particle hits is necessary when analyzing the effect of low-fluence ion-beam radiation. As shown above, our irradiation system has the ability to determine the numbers and the positions of ion hits with quite high accuracy by using a real-time etching method of ion track detection. Thus, for determining the cellular effects of low-dose high-LET radiation, our heavy-ion microbeam system will be a useful tool.

The cells that were only hit at their cytoplasm also showed limited cell growth. This appears to reflect the cytoplasmic effect of the particle radiation. However, in the case of high-energy particle radiation, the range of the δ electrons is increased compared to that of particles of lower velocities. This means that there is a possibility that this limited cell growth might be caused by the effects of the δ electrons on the cell nucleus, even if the core of the ion-beam track only traverses the cytoplasmic region. Further investigation is needed to determine whether this growth inhibition is caused by the direct effect of an ion hit on the cytoplasm, through a cytoplasmic effect, or by the effect of δ-electron diversion of the penumbra of the ion-beam track. Recently, we established a method for measuring the DNA damage induced in individual cells (10). This method will enable us to determine the answer to above question and will also show us the quantitative relationship of the ion hit position to the yield of DNA damages.

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Improvement of the GCI X-Ray Microprobe and Bystander Studies Using V79 Cells

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Current studies of radiation effects in cellular systems have found a range of responses which predominate at low doses and low dose rates (1, 2). Among them, the bystander responses have attracted considerable interest in recent years, and several investigators have reported a binary behavior, with the effect triggered by very small doses and immediately reaching a plateau phase. In spite of the large number of experiments performed, the mechanisms that trigger such effects and the factors that regulate them are still unknown, and more experiments are needed. The Ultrasoft X-ray Microprobe in operation at the Gray Cancer Institute is a facility designed to assess the biological response of individual cells *in vitro* irradiated with a low-energy carbon X-ray beam (278 eV) focused into a sub-micrometer spot (<0.5 μm in diameter) (3). Recent improvements have been made to upgrade the facility with higher-energy X rays such as aluminum K_α (1.47 keV) and titanium K_α X rays (4.5 keV). The use of higher-energy X rays will provide a unique powerful tool to investigate a wide range of radiobiological phenomena. The different absorption properties of characteristic K_α carbon, aluminum and titanium X rays with biological tissues will produce different energy deposition patterns within the samples. After the irradiation of a typical mammalian

cell (V79) with a focused beam of carbon X rays, about 35% of the dose delivered will be absorbed by the first layer of cytoplasm ($\sim$0.7 μm, as measured using a confocal microscope). The dose through the sample will then decrease exponentially, with negligible dose being absorbed at the other side of the cell. In contrast, titanium X rays will deposit only 6% of the dose in the cytoplasm, producing basically a constant dose absorption pattern throughout the entire sample. Moreover, the secondary electrons produced by the absorption of carbon, aluminum or titanium X rays will have different energies that produce different ionization patterns that could extend from 7 nm (C_K X rays) up to several hundred nanometers (Ti_K X rays). In this way, some characteristics of both the high- and low-LET radiations could be mimicked and the relevance of clusters of DNA damage addressed precisely. Finally, since X rays do not suffer from scattering, small targets several hundreds of micrometers inside biological tissues could be targeted with sub-micrometer precision, opening the possibility of tissue experiments using microbeams.

Physics improvements have been concentrated on the X-ray focusing elements (i.e. zone plates). Zone plates of different sizes and characteristics have been designed to be used with the existing settings and to maximize the potential of the X-ray source according to the energy to be used. Preliminary measurements have clearly confirmed the theoretical calculations, and zone plates of Si_3N_4 (400 μm diameter), germanium (300 μm diameter) and tungsten (100 μm diameter) are respectively used to successfully focus the C_K, Al_K and Ti_K X rays. The focusing efficiencies of such lenses drops rapidly with increasing X-ray energy (from 14% for C_K to $\sim$3% for Ti_K) as expected but still provides useful dose rates for biological experiments (entrance dose rate for a typical mammalian cell of $\sim$2 Gy/s for C_K and Al_K X rays and $\sim$10 mGy/s for Ti_K X rays). A final X-ray focal spot of sub-micrometer size is still achieved for all the energies available with negligible background radiation reaching the biological samples. Measurements of the characteristic X-ray production by electron bombardment of thick targets have also been performed to identify the best running conditions for the X-ray source. Such measurements indicate a peak for X-ray production between electron energies of 15 and 20 keV since higher-energy electrons will penetrate deeper into the targets and the photon produced will be reabsorbed by the target itself. On the other hand, the linear increase in X-ray production as a function of the current of electrons striking the target provides a very easy and reliable method for controlling the dose rate during the experiments. The present electron current is limited to a few hundred electron volts, but future improvements are planned to increase it, possibly through cooling and/ or rotating targets.

Although the recent improvements have upgraded the facility to include Al_K and Ti_K X rays, most of the bystander studies have been performed by irradiating V79 cells with C_K X rays of 278 eV. The high sensitivity provided by the accurate irradiation and revisiting of the individual samples allowed us to address specific aspects of the bystander phenomenon in a unique way. In particular, evidence of a dose dependence of cell killing by bystander effects has been found at doses below 0.2 Gy, where no difference is observed between all-cell and single-cell irradiation. The effect then reaches a plateau of about 10% for higher doses. The extent of the bystander effect appears to be independent of the number of cells irradiated (up to 5) when the surviving fraction is plotted as a function of the dose delivered to the single cell(s). Interestingly, the same data plotted as a function of the total amount of energy delivered to the whole cell population indicate an increased effect since it concentrates the energy into a single sample.

This whole population behavior is in contrast with that found at the single-cell level, where no statistically significant differences have been found in the surviving fraction of a population in which each sample has been irradiated with a C_K X-ray beam of 0.5 and 10 μm. Although even in the case of irradiation with the 0.5-μm beam no significant temporal and spatial overlaps of tracks are expected, no differences are detected whether the direct damage from the radiation is concentrated in a small volume ($\sim$2 μm^3) or spread over the whole cell. This result appears to indicate that there are different mechanisms through which bystander ef-

fects and the direct action of radiation affect the cell response. The different nature of the damage that is induced is also highlighted by the different responses to radical scavengers such as DMSO. Clonogenic experiments in the presence of 8% DMSO (pre- and postirradiation) clearly show that the radical scavenger does not affect the bystander response significantly while protecting the population in the case of all-cell irradiation, in agreement with findings reported by others using conventional irradiation techniques (4).

The ultrasoft X-ray microprobe has also been used to investigate the relevance of targets other than the cell nucleus in the bystander response. By using a differential seeding technique, our single cell survival assay protocol (5) has been improved for cytoplasmic irradiation. By seeding only a few cells several hours before the irradiation in a remote corner of the cell dish, it was possible to manually irradiate their well-spread cytoplasm without delivering any radiation to the nucleus. The survival analysis was then assessed on the single cells plated only a few hours before the irradiation in the center of the dish. After the irradiation of the cytoplasm of a single cell, the clonogenic potential of the whole cell population is reduced by the same amount as for the nuclear irradiation. In both irradiation cases, a plateau response of about a 10% decrease in survival is achieved independent of the dose delivered. By considering that also during the nuclear irradiation with carbon X rays an extra 35% of energy is absorbed by the cytoplasm, the data appear to point to cytoplasmic damage as a critical factor in triggering the bystander effect. Further experiments using titanium X rays (only 6% of the dose delivered is absorbed by the cytoplasm) are planned to investigate the relevance of DNA damage to the bystander phenomenon more precisely.

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Session VII

Chair: A. Chatterjee

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Energy Deposition Patterns and the Bystander Effect

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Most radiation exposure resulting from industrial, research and medical use of radiation is low LET, primarily the secondary electrons produced by X and γ rays. Consequently, it is important to consider both low-LET and high-LET radiation when investigating the biological consequences

of low-dose and low-dose-rate irradiation. The frequent occurrence of radiation-induced response in unirradiated individuals within a population of cells receiving a low dose of high-LET radiation (the bystander effect) suggests that cells do not act independently when expressing the stochastic effects of high-LET radiation. Whether the consequence of the bystander effect is an increase or a decrease in the probability of a given health effect is not yet known, but it is clear that it increases the effective target size, and it therefore reduces the dose below which a linear extrapolation to zero dose must be valid. Since the bystander effect is evidently significant for high-LET radiation, it seems necessary to ask if it is also significant for low-LET radiation. To address this question, we have built a simple electron source covering the range of 0 to 100 keV and equipped it with a collimator for irradiation of selected areas within a growing cell population.

We have conducted experiments intended to detect a bystander effect due to exposure to 50 to 70 keV electrons using a variety of cell lines. Some of these lines were selected because they had been shown to demonstrate a bystander effect for high-LET radiation; others were selected because they were more nearly representative of the types of cells frequently associated with the initial stages of the development of cancer. Fluorescence assays for CDKN1A and PCNA were used for a relatively quick and quantitative evaluation of damage in bystander cells. Measurements with α-particle irradiation were used to confirm that the bystander effect was occurring with high-LET radiation. For the low-LET radiation, electrons with energies near 50 keV were used to irradiate all cells in a narrow strip across the center of a cluster of cells approximately 1 cm in diameter. A bystander effect was evident in AG 1522 human fibroblast cells, in clone 9 rat liver cells, in RIE mouse intestinal cells, and in NTEC rat primary tracheal cells. The only group of cells which we have tested and have not found a bystander effect for high-LET radiation is primary human bronchial cells, which appear to begin differentiation very quickly. On the other hand, we have not been able to detect elevated CDKN1A or PCNA in any unirradiated cells in populations where about 10% of the cells received a high number of electron tracks. That is, we could not find a bystander effect in AG 1522, clone 9, RIE or HBEC cells exposed to 50 keV electrons.

Since we have been unable to show a bystander effect due to medium-energy electrons, even in cell lines that generally show bystander effects in our experiments with α particles and that have demonstrated bystander effects for ultrasoft X-ray experiments (Schettino, Newman, Prise, Folkard, Held and Michael, 49th Annual Meeting of the Radiation Research Society, April 2002), we have started searching for the experimental difference that is responsible for the divergent results. It is possible that some end points will show a significant bystander effect for high-LET (α-particle) radiation but not for low LET, while other end points (such as micronucleus induction) may be more sensitive to whatever signal is producing bystander effects, and may therefore be sensitive to both high- and low-LET radiation. Additional experiments are being conducted to broaden the range of end points studied to check this possibility. We are also beginning to consider differences in the energy deposition patterns for different microbeam irradiation modalities. There are clearly differences in the energy deposition between heavy ions, fast electrons, and soft X rays, and these differences will result in different distributions of chemical changes within the irradiated cells. However, it is not clear how to characterize these differences in energy deposition most effectively. Since we do not have a clear indication of the mechanisms involved in the bystander effect, there is no basis for selecting one measure of radiation quality. However, it is possible to discuss similarities and differences between the energy deposition patterns of the three modalities.

In each form of microbeam irradiation, much of the energy is deposited by low-energy electrons. In the case of soft X rays, all energy is deposited by electrons with energies equal to or less than the X-ray energy. For moderate-energy (order of 50 keV) electrons, the secondary electron (δ-ray) distribution builds up very quickly and the total number of electrons producing ionizations is several orders of magnitude more than the num-

ber of incident electrons. Although production of δ rays with energy up to half the energy of the primary is possible, most δ rays are low energy, and a typical electron track can be visualized as a forward biased random walk surrounded by a sparsely populated field of branching low-energy electron tracks. In a typical equilibrium spectrum there are about 100 times as many electrons at 100 eV as there are at 10 keV. Positive ion tracks are typically viewed as a line of ionization produced directly by the ion, surrounded by a region with numerous δ-ray tracks. The number of δ rays is large; the majority have relatively low energy, and they are responsible for about half of the total ionization produced by the high-LET particle, so they constitute a large number of electron track ends. The logical conclusion from these general observations is that the majority, if not all, of the energy deposited by any of the microbeam irradiation systems is deposited by electrons near the ends of their tracks, that is, by electrons with energies of a few hundred electron volts or less. If this is true, and if the moderate-energy electrons are much less likely to produce bystander effects than are 5 MeV α particles, the cause must lie with the spatial or temporal distribution of those low-energy electrons.

In most experiments we find that a single α particle through a cell is enough to trigger bystander effects in neighboring cells. Assuming a cell is about 7 μm thick in the direction of the beam, the α particle deposits of the order of 700 keV when it traverses the cell. To deposit the same amount of energy would require 2,500 carbon K-shell X rays or about 150 electrons with a stopping power of 0.65 keV/μm (50 keV). To determine the scope of the energy distribution problem, we can apply the extreme simplification of assuming that the electron track ends that are responsible for the majority of the energy deposition by the α particle occurs in a 20-nm-diameter cylinder along the geometric path of the α particle. Thus these track ends occur randomly within a volume of about 2.2×10^{-3} μm³. In the case of the ultrasoft X-ray microbeam (1), we will assume that the X-ray interactions in the cell are confined to a volume 0.5 μm in diameter and 3.5 μm long, since only about 12% of the X rays penetrate beyond the midplane of the cell. In this case the electron track ends are confined to a volume of about 0.69 μm³. Finally, in the case of the moderate-energy electron microbeam, we can assume that the track ends occur within 10 nm of the geometric path of the fast electrons, and that, due to electron track branching, 150 incident electrons will generate about 2000 μm of energetic electron track within the typical cell. The result is that the moderate-energy electrons produce track ends within a total volume of about 0.63 μm³. Surprisingly, this is essentially the same irradiated volume as that produced by the ultrasoft X rays. However, there is a very big difference between the X and electron irradiations. The electron track ends produced by X rays are all within a single compact cylindrical volume occupying only about 0.017% of the volume of the cell (0.14% of the volume of the cell nucleus). In contrast, the track ends produced by electrons are distributed along the tracks which, due to multiple scattering, are distributed nearly uniformly throughout the volume of the cell (2). This difference becomes significant if triggering the cellular reaction requires more than a minimum concentration of damage in a volume larger than a few base pairs in DNA. For example, there are 0.5-μm-diameter spheres within an α-particle-irradiated nucleus that have as much as 50 keV deposited in them. In the case of the carbon K-shell X rays, some 0.5-μm-diameter volumes have an energy deposition of more than 66 keV. However, the maximum energy deposition in a 0.5-μm-diameter region of a cell irradiated by moderate-energy electron microbeam is probably not much more than 0.3 keV.

It appears that, assuming that the event which triggers a cell to produce a signal which results in a bystander effect is more complex than a single strand break or a single base change in DNA, the difference in the energy deposition patterns of α-particle tracks, ultrasoft X-ray microbeams, and moderate-energy electrons could result in a large difference in the induction of bystander effects. Since we do not yet know what type of damage is needed to trigger a bystander effect, we cannot immediately select the appropriate site size for dosimetry evaluations, but we may be able to use the energy deposition characteristics of radiations that do and do not produce bystander effects to help define the limits of processes that may be relevant.

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Interaction of Radiation-Induced Adaptive Response and Bystander Mutagenesis in Mammalian Cells

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There are many reports on the role of the bystander effect and the adaptive response, two interesting and important phenomena, in low-dose radiation effects (reviewed in refs. *1–5*). The bystander effect tends to exaggerate the effect of low doses by eliciting the damage in nonirradiated cells, while the adaptive response dampens damage to a subsequent challenging dose after a low initial priming dose. Although these two conflicting phenomena have attracted much interest, there are very few data that address the interaction of the two effects (*6–8*).

Using the Columbia University charged-particle microbeam and the highly sensitive A_L cell mutagenic assay, we reported previously that cells that had been lethally irradiated with α particles could induce mutagenesis in neighboring cells not directly hit by the particles, and that reactive oxygen species were not directly involved (*9*). Furthermore, the findings can also be extended to a single α-particle irradiation, and gap junction-mediated cell–cell communication plays an important role in the process of bystander mutagenesis (*10*). In our present study, two related experiments were designed to explore the interaction of bystander effects and adaptive response. First, we addressed the question whether low-dose radiation decreased bystander mutagenesis. A_L cells were plated in specially constructed microbeam dishes 2 days before treatment as described previously (*9, 10*). After exposure to graded low doses of X rays, 4 h later, 10% of the cells (selected randomly) were irradiated with either a single or 20 α particles using the Columbia precision particle microbeam. In our second series of experiments, we examined the cytotoxic and mutagenic response of the bystander cells to a subsequent high-dose radiation. Ten percent of randomly selected cells in the microbeam dishes were irradiated with a lethal dose of 20 α particles. Four hours later, cultures were irradiated with a subsequent challenging 3-Gy dose of X rays. After the second irradiation, the cells were maintained in microbeam dishes for 2 days before being replated in culture flasks. Determination of the mutant fraction and mutant spectrum analysis were carried out as described before (*11–13*).

We found that the bystander mutant yield induced in a population where 10% of randomly selected cells were irradiated with a single α particle decreased significantly if the cells were pretreated with either 0.02 or 0.1 Gy of X rays. Pretreatment with a 0.1-Gy dose of X rays decreased the bystander mutagenic effect induced by a single and 20 α particles by 60% and 30%, respectively. Furthermore, an increase in the priming dose decreased the inhibitory effect such that pretreatment with 0.5 Gy X rays reduced the bystander mutant yield by only 12%; the difference was not statistically significant. The results indicate that in the presence of low-dose radiation stress, bystander mutagenesis is suppressed by the adaptive response. Although the mechanism(s) is unclear,

it is likely that when cells are exposed to low-dose X rays, they initiate a series of self-preservation mechanisms that diminish their ability to respond to bystander signaling.

To determine the response of bystander cells to a challenging dose of radiation, 10% of randomly selected cells were irradiated with a lethal dose of 20 α particles each. Four hours later, cultures were irradiated with 3 Gy of X rays. We found that bystander cells that were not directly hit by α particles had a significantly higher mutant yield than control cells exposed to X rays under similar conditions ($P < 0.05$). Furthermore, the mutant yield for irradiated bystander cells was significantly higher than a simple additive effect of bystander mutation and X rays ($P < 0.05$). These data indicate that bystander cells show an increase in sensitivity after a subsequent challenging dose of X rays. Results from the present study imply that the radiobiological effect at low dose is a complex interplay between the adaptive response and the bystander effect. These results address some of the fundamental issues regarding both the actual target and the radiation dose effect and can contribute to our current understanding for radiation risk assessment.

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Effect of Cell Density on the Magnitude of the Bystander Response

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Previous work using the Columbia University microbeam has demonstrated a reproducible bystander effect for the end points of clonogenic survival and oncogenic transformation using C3H 10T½ cells (*1*). The aim of the present study was to assess the relative importance of gap junction communication between cells compared with transfer of cytokines through the medium as mediators of this bystander effect. To facilitate this, C3H 10T½ cells were plated at high and low cell concentrations and irradiated with high-LET α particles.

Materials and Methods

Approximately 18 h prior to irradiation, exponentially growing C3H 10T½ cells (passage 9–12) were plated onto 3.8 μm polypropylene in the center of a series of 6.3-mm-diameter miniwells. Cells were plated to give a final concentration of either 200 or 2000 cells per dish. When cells were viewed through the microscope, the lower concentration resulted in isolated cells with very little cell-to-cell contact compared to the higher concentration, where cells were approximately 90% confluent, allowing direct communication. The Columbia microbeam system and the irradiation procedure have been described in detail elsewhere (*2*). Briefly, the attached cells were stained for 0.5 h with a low concentration of Hoechst 33342, enabling individual nuclei to be identified and located with the optical image analysis system. Immediately prior to irradiation, cells were washed with serum-free medium to avoid fluorescence from serum components, and irradiations were carried out in the presence of a thin film of serum-free medium. Then either 10% or 100% of the cells were irradiated with 6 MeV α particles over a range of 2 to 12 α particles per cell. The average stopping power of the α particles traversing the cells was 90 keV/μm. After irradiation, the cells were washed twice with PBS, trypsinized and replated at a low density of about 100 viable cells into 100-mm culture dishes and incubated for 2 weeks. Cells were then stained with 2% crystal violet and the resulting colonies (containing greater than 50 cells) were scored to determine plating efficiencies and surviving fractions of the control and irradiated cells.

To assess oncogenic transformation, 10% of the cells were irradiated with eight α particles, and approximately 300 viable cells were plated into 100-mm culture dishes. The cells were incubated for 7 weeks with culture medium replaced every 12 days before being fixed and stained with Giemsa to identify morphologically transformed type II and III foci, as described elsewhere (*3*).

Data from a minimum of three independent experiments were pooled. All data for clonogenic survival are presented as mean values.

Results and Discussion

As would be expected, significant cell killing was seen when 100% of the cells were irradiated, with survival falling to approximately 0.27 at 12 α particles per nucleus. It is possible to calculate the percentage of cells that would be expected to survive when 10% of the cells are exposed to various numbers of α particles in the absence of any bystander effect by applying the curve for 100% survival to the 10% of cells that were actually hit. Therefore, in the absence of a bystander effect, we would expect survival of 0.93 at a dose of 12 α particles/cell. At both cell densities, the surviving fraction falls progressively as the number of α particles delivered per cell is increased and the survival observed is lower than that expected in the absence of a bystander effect. The fact that a bystander effect is evident even at the low cell concentration suggests that it is not wholly dependent on cells being in direct contact. However, survival at the higher concentration is significantly lower than that at the

low concentration ($P < 0.001$), with a surviving fraction at a dose of 12 α particles of 0.76 compared to 0.88. Considering oncogenic transformation, a higher transformation frequency was observed when cells were irradiated at high density ($9.32/10^4$ surviving cells) than at low density ($4.97/10^4$ surviving cells). These data suggest that when a cell is hit by radiation, a signal is transmitted through cell-to-cell contact to neighboring unirradiated cells, resulting in a larger bystander effect.

To further assess the importance of cell-to-cell communication in mediating the observed bystander effect, we pretreated the cells with Filipin. Filipin disrupts lipid rafts present in the cell membrane, thereby inhibiting membrane signaling and interfering with gap junction-mediated intercellular communication. Cells plated at both high and low density were incubated with Filipin at a concentration of 1 μg ml^{-1} for 1 h at 37°C, and 10% of the cells were then irradiated on the microbeam with 12 α particles per cell. When cells were irradiated at low density, preincubation with Filipin had no effect on cell survival (SF of 0.90 ± Filipin). However, at high cell density, pretreatment of the cells with Filipin caused an increase in cell survival similar to the level seen in the low-density cultures [SF of 0.87 (+ Filipin) compared to 0.74]. This is further evidence for the importance of the cell membrane in the transmission of signals from hit cells to bystander cells.

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Involvement of Nitric Oxide in the Bystander Responses Induced by Microbeam-Targeted Cells

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Recently, considerable evidence has been accumulated in support of the existence of a bystander effect, where cells having received no irradiation show biological consequences from their neighboring irradiated cells. This phenomenon was first reported by Nagasawa and Little (*1*) and then was demonstrated by other studies with various biological end points including cell killing, micronucleus (MN) induction, mutation induction, and alterations in cell growth. It is believed that some medium-derived soluble factors such as short-lived reactive oxygen species (ROS) (*2*) and long-lived transforming growth factor β1 (TGFB1) (*3*) could be released from irradiated cells and then further induce a series of bystander responses in the nonirradiated neighboring cells. We have demonstrated that nitric oxide (NO) also contributes to the medium-mediated bystander effect (*4, 5*).

The application of single-cell microbeam facilities, in which cells are irradiated individually with a predefined exact number of particles, is providing new insights into the radiation-induced bystander effect (*6–8*). A bystander mutagenic effect has been found in nontraversed cells when a proportion of mammalian cells have suffered a precise number of nuclear traversals by α particles (*9*). Belyakov *et al.* (*10*) reported that when a single cell within a population was targeted by one helium particle, an additional 100 damaged cells typically could be observed in the surrounding nonexposed population.

In the present study, cells of a radioresistant human glioblastoma cell line T98G and a primary human fibroblast cell line AG01522 were used. It was found that when a fraction of the T98G cells that have been sparsely seeded on the Mylar film-based microbeam dish were individually targeted with a precise number of helium ions generated from the Gray Cancer Institute Charged Particle Microbeam, micronucleus induction significantly exceeded the expected value calculated from the number of micronuclei observed when all the cells were targeted, assuming that no bystander effect occurred. Even when only a single cell within a population was hit by one helium ion, the MN induction in the population could be increased by about 20% 1 h after irradiation. These results provide direct evidence of a radiation-induced bystander effect. Moreover, the medium harvested from microbeam-targeted T98G cells showed a cytotoxic effect when assayed by MN induction in unirradiated T98G cells.

In other experiments, T98G cells and AG01522 cells were co-cultured in two separate regions of the same microbeam dish. When a fraction of the T98G cells were individually targeted with counted helium ions, followed by 24 h of cell co-culture, the MN induction in the unirradiated adjacent AG01522 population was increased by about 70%. In contrast, when a fraction of the AG01522 cells were individually targeted with helium ions, the MN induction in the unirradiated T98G population was also increased, but by only about 20–30%. A more interesting finding was that the increase in MN induction in the unirradiated population was independent of the number, from one cell to 100% of the population, of targeted co-cultured cells, and that this increase was independent of the number of the helium ions delivered to the targeted cells. These results indicate that the radiation dose itself is just a trigger of the bystander response and that a signal molecule is then secreted from the irradiated cells which contributes to the bystander response.

To investigate the factor involved in this bystander effect, we treated cells with c-PTIO, a NO-specific scavenger, or aminoguanidine, an inhibitor of iNOS during irradiation and subsequent co-culture. Both of these treatments reduced the bystander MN induction to the expected low level in the irradiated T98G population and to the background levels in (1) the unirradiated T98G cells treated with the medium harvested from irradiated cells and (2) the unirradiated T98G cells co-cultured with irradiated AG01522 cells. However, the c-PTIO treatment only partly decreased MN induction in the unirradiated AG015522 cells co-cultured with irradiated T98G cells. Consequently, NO plays an important signaling role in the microbeam radiation-induced bystander effect in T98G cells with a mutant type of TP53, and other signaling molecules such as ROS may contribute to the radiation-induced bystander effect in AG01522 cells with wild-type TP53.

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Session VIII

Chair: L. Braby

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How Many Bystander Effects Are There?

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The bystander effect is not new. As early as the 1940s, there were reports that the inactivation of biological entities may be brought about equally by ionizations produced within the entity or by the ionization of the surrounding medium (*1–4*). By 1947, Kotval and Gray (*5*) had shown that α particles which pass close to the chromatid thread, as well as those which pass through it, have a significant probability of producing chromatid and isochromatid breaks or chromatid exchanges.

The term used today to describe such phenomena is the bystander effect, a name borrowed from the gene therapy field, where it usually refers to the killing of several types of tumor cells by targeting only one type of cell within a mixed population (see ref. *6*, for example).

In the field of radiation biology, it has come to be loosely defined as the induction of biological effects in cells that are not directly traversed by a charged particle but that are in proximity to cells that are traversed.

Data now available concerning the bystander effect fall into two quite separate categories, and it is not certain that the two groups of experiments are addressing the same phenomenon. First, there are experiments involving the transfer of medium from irradiated cells, which results in a biological effect in unirradiated cells. Second, there is the use of sophisticated single-particle microbeams, which allow specific cells to be irradiated and biological effects to be studied in their neighbors; in this case communication is by gap junctions.

Medium transfer experiments have shown bystander effects for cell lethality, chromosomal aberrations and cell cycle delay. The type of cell, epithelial or fibroblast, appears to be important, though data are conflicting. Experiments suggest that the effect is due to a molecule secreted by irradiated cells which is capable of transferring damage to distant cells. Use of a single microbeam has allowed the demonstration of a bystander effect for chromosomal aberrations, cell lethality, mutation and oncogenic transformation. When cells are in close contact, allowing gap junction communication, the bystander effect is much greater than the phenomenon demonstrated in medium transfer experiments.

Evidence comes from experiments with V79 cells, where the end point observed was cell lethality. Lines of hygromycin- and neomycin-resistant V79 cells were produced (*7*). Before exposure, the hygromycin-resistant cells were stained with a low concentration of a vital nuclear dye and then plated in microwells in the proportion of nine neomycin-resistant cells for every one hygromycin-resistant cell. Using the Columbia microbeam, the computer was programmed to irradiate only the 10% of cells stained with a nuclear dye with various numbers of α particles from 1 to

16, aimed at the centroid of the nucleus. The cells were then removed and cultured for survival in the appropriate growth medium, which made it possible to obtain survival curves for hit and non-hit cells. There was a considerable amount of cell killing in the non-hit cells, implying a substantial bystander effect. The magnitude of the bystander effect in these studies is much greater than that reported by the group at The Gray Cancer Institute, where only 5 to 10% lethality was seen in non-hit cells, using protons or soft X rays in a microbeam. The difference is probably accounted for by the cell density. In The Gray Cancer Institute studies, only about 200 cells were seeded in an area of 10×10 mm. The average distance between cells was therefore some hundreds of micrometers, so it is likely that communication via gap junctions did not contribute to the effect observed (B. D. Michael, personal communication). By contrast, in the Columbia studies, 1,000 to 1,200 cells were plated in a miniwell 6.3 mm in diameter so that 50 to 60% were in contact, allowing gap junction communication, which has been demonstrated to be important in mutation studies with the microbeam. Therefore, these data support the notion that communication through the medium and communication through gap junctions are separate phenomena, because the magnitude of the effect is so different.

A very large bystander effect was observed in studies of oncogenic transformation in C3H 10T½ cells, where, to have sufficient cells for this assay, cells were plated at high density and therefore were in gap junction communication (8). In these experiments, mouse fibroblast (C3H 10T½) cells were plated in a monolayer, and the computer was programmed to irradiate either every cell or every tenth cell selected at random with one to eight α particles directed at the centroid of the cell nucleus. The cells were subsequently removed by trypsinization and replated at low density, and transformed foci were identified 6 weeks later by their morphological appearance.

The data show that: (a) More cells can be inactivated by α particles than were actually traversed by an α particle. (b) When 10% of the cells on a dish are exposed to two or more α particles, the resulting frequency of induced oncogenic transformation is indistinguishable from that when all the cells on the dish are exposed to the same number of α particles.

One of the few true comparisons of bystander effects observed by the putative transfer of cytotokines through the medium rather than by gap junction communication in closely associated cells comes from the work of Zhou and colleagues (9, 10). They studied mutation in A_L cells by either irradiating densely cultured cells with the Columbia microbeam or irradiating cells on one surface of a double Mylar dish and observing as bystanders the cells on the other side of the dish. Their results suggest that the cytotoxic factor(s) released from cells into the culture medium had a small, barely significant effect on the mutagenic response of the nonirradiated cells. In contrast, the microbeam-based bystander studies show a threefold elevation of mutation incidence in bystander cells.

Conclusion

We conclude that for three different model systems involving cell lethality, mutation and oncogenic transformation, the bystander effect seen when cells are in close gap-junction contact is much larger than the effect resulting from the transfer of cytokines through the medium. This survey strongly supports the notion that there are at least two quite separate bystander effects.

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Studies of Bystander Responses with the GCI Microbeams

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The original rationale for the development of the current generation of microbeams was based on studying the localized deposition of low doses of radiation in subcellular locations. Much of the recent effort, however, has focused on studying interactions between hit and non-hit cells, with particular emphasis on the bystander response. These studies have contributed to a sea change in basic concepts regarding the interactions of radiation with cells and tissues, particularly at low doses. Our own studies have used both a charged-particle microbeam (1, 2) and a focused soft X-ray microprobe (3) developed at the Gray Cancer Institute to probe a range of subcellular, cellular and tissue-dependent processes. The ability to test for responses to individual charged-particle or electron tracks is important for determining whether relevant biological changes can be induced by these low doses and for evaluating their contribution to the overall response of a cell population or tissue after irradiation.

Targeted Studies

Significant evidence has already been reported for the role of non-nuclear targets in radiation effects leading to mutation using targeted helium ions (4). Our own recent studies have shown that cytoplasmic irradiation can trigger bystander responses. Using both microbeams, we have compared the effects of individual charged particles or soft X rays targeted through the cytoplasm relative to that observed by targeting the nucleus. When cell killing is measured, no difference is observed in the level of bystander response, confirming that the response is not due to direct DNA damage. This is observed for a single 3 MeV helium ion delivered to a single cell within a population or targeted C_K photons at doses as low as 200 mGy. Also, the response is observed in both human and mouse fibroblasts. For carbon-K soft X rays, most of the energy deposition occurs in the first micrometer of the cell. One suggestion from these studies is that it may be membrane sites that are important targets, as suggested by recent published data (5).

Interactions between Targeted and Non-targeted Responses

Bystander responses are classified as non-targeted effects, and there have been some suggestions that many of these responses may be related.

In pilot studies we have been comparing interactions between bystander responses and conventional exposures to test their role in adaptive processes. The effect of treatment of cells with conventional doses before or after a bystander treatment where only a single cell was targeted was examined for a range of doses. Importantly, significant interactions were observed between the two treatments. At low doses, when a bystander treatment is given, followed 1 h later by a conventional dose of X rays, significant protection is observed even after X-ray exposures as low as 20 mGy. The overall response, however, is determined by the relative doses delivered by the conventional or bystander treatment and the order in which they are given. This suggests that there is a complex interplay between the two processes which governs overall response.

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Studies of Bystander Effects in 3D Human Tissue Systems: What Can They Mean?

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The bystander effect involves cells directly hit by radiation sending out signals to adjacent non-hit cells. These signals have been demonstrated to increase the probabilities of a variety of different end points. It has been suggested that bystander effects may well dominate radiation effects at very low doses. Most studies to date have focused on two-dimensional monolayers, often with cells that are not in contact. We are studying radiation-induced bystander effects in three-dimensional human tissue systems. To maintain good reproducibility, we are using several novel artificial human skin tissue systems. These artificial tissues allow us to model conditions present *in vivo*. Three systems are being used to study bystander responses: a model of the normal human epidermis, a corneal human model, and a model that reconstructs the tracheal/bronchial epithelial tissue of the human respiratory tract. The Radiological Research Accelerator Facility (RARAF) microbeam is being used to irradiate tissue samples in a known pattern (well-separated points on a line, accuracy ± 2.5 μm) with a known number of particles. After irradiation, the tissue is incubated, embedded in paraffin, and then cut into 5-μm histological sections located at varying distances from the plane of the irradiated cells. We are using three end points: an *in situ* apoptosis assay, epidermal differentiation, and a proliferation assay. In this three-dimensional system, we have demonstrated a clear bystander response.

What do these 3D bystander effects imply? Radon risks derive from exposure of bronchio-epithelial cells to α particles. Alpha-particle exposure can result in bystander effects, where irradiated cells emit signals resulting in damage to nearby unirradiated bystander cells. Bystander effects can cause downwardly curving dose–response relationships and inverse dose-rate effects. We have extended a quantitative mechanistic model of bystander effects to include protracted exposure, with inverse dose-rate effects attributed to replenishment, during exposure, of a subpopulation of cells which are hypersensitive to bystander signals. In this approach, bystander effects and the inverse dose-rate effect are manifestations of the same basic phenomenon. The model was fitted to data on radon-exposed miners, which show dose- and dose-rate-dependent effects. The results suggest that one directly hit target cell can send bystander signals to about 50 neighboring cells and that, for domestic radon exposures, the risk could be dominated by bystander effects. The analysis concludes that a naïve linear extrapolation of data from radon miners to predict radon effects at low doses without accounting for dose-rate/bystander effects would result in an underestimation of domestic radon risks by about a factor of ~4. However recent domestic radon risk estimates (BEIR VI) have already applied a phenomenological correction factor of ~4 for inverse dose-rate effects and have thus already implicitly taken into account corrections that we suggest here are due to bystander effects. Thus current domestic radon risk estimated are unlikely to be underestimates as a result of bystander effects.

Microbeam and Low-Fluence Dosimetry

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In a biological context, low-dose radiation exposures might be defined as those in which cells are subject to the passage of small numbers of charged-particle tracks—say, an average of 1 track or less per cell. These tracks may be primary, as with charged-particle irradiation, or secondary, as with X rays or α particles or with neutrons. Microbeam irradiation generally operates in the low-dose region and constrains energy deposition in terms of the number of events and their location. In these situations, dosimetric concepts have to be applied with particular care. The first priority with microbeam exposures is to specify them in such a way that sufficient information is provided that they could be repeated at another installation. This information has to include a description of the morphology of the cells. Other considerations include how to make meaningful intercomparisons of the effects of different radiation qualities or of targeting different regions of the cell. These are some of the main requirements in the laboratory, but in addition there is a need to consider how best to describe these low-fluence exposures in that terms may ultimately be useful toward improved evaluation of low-dose risk.