X-ray-induced bio-acoustic emissions from cultured cells

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ABSTRACT
Purpose: We characterize for the first time the emission of acoustic waves from cultured cells irradiated with X-ray photon radiation.
Methods and materials: Human cancer cell lines (MCF-7, HL-60) and control cell-free media were exposed to 1 Gy X-ray photons while recording the sound generated before, during and after irradiation using custom large-bandwidth ultrasound transducer. The effects of dose rate and cell viability were investigated.
Results: We report the first recorded acoustic signals captured from a collective pressure wave response to ionizing irradiation in cell culture. The acoustic signal was co-terminous with the radiation pulse, its magnitude was dependent on radiation dose rate, and live and dead cells showed qualitatively and quantitatively different acoustic signal characteristics. The magnitude of the collective acoustic peaks was temporally wider and with higher acoustic power for irradiated HL-60 than for irradiated MCF-7.
Conclusions: We show that X-ray irradiation induces two cultured cancer cell types to emit a characteristic acoustic signal for the duration of the radiation pulse. The rapid decay of the signal excludes acoustic emissions themselves from contributing to the inter-organism bystander signal previously reported in intact animals, but they remain a potential component of the bystander process in tissues and cell cultures. This preliminary study suggests that further work on the potential role of radiation-induced acoustic emission (RIAE) in the inter-cellular bystander effect is merited.

Introduction
The observation that the 'post-irradiated state', comprising genomic instability and delayed cell death amongst other cell-type dependent phenomena, can be passed from an irradiated cell, tissue, or whole organism to one nearby, raises the important question of how the information might be transferred (Mothersill and Seymour 2019). How the radiation-induced bystander effect (RIBE) is initiated and transferred (Mothersill and Seymour 2019). In further experiments reported in the same paper light signals were blocked using aluminum foil and attenuation calculations performed to check if a bio-acoustic signal could be the mediator. It could not be excluded as the distances and acoustic characteristics of the intervening water and extracellular material are likely to provide efficient sound transmission but were not proven. In a review of the literature in the field, we considered the possibility that an acoustic signal might be involved with the initiation or propagation of the bystander signal (Matarèse et al. 2022).

There is an intrinsic relationship between electromagnetic forces (mediated by photons) and acoustic vibration (mediated by phonons), experimentally established in the 19th century (Bell 1881). On exposure to ionizing photons, inner-shell electrons are excited, generating photoelectrons, Auger electrons, or electromagnetic radiation (e.g. photoluminescence), which decay producing cascades of secondary electrons as these decay processes transfer kinetic energy to...
surrounding atoms to reach thermal equilibrium. These processes lead to a transient thermoelastic expansion of the biological structure generating the pressure waves of an acoustic emission (Garcia et al. 1988).

We recently proposed the hypothesis that acoustic energy released on interaction of biota with electromagnetic radiation may represent a signal released by irradiated cells leading to, or complementing, or interacting with, other responses, such as endosome release, responsible for signal relay within the unirradiated individuals in the targeted population (Matarèse et al. 2022). In this study, the primary objective was to experimentally demonstrate, for the first time, the bio-acoustic wave emissions generated as biological cellular response during X-ray photon irradiation of cells in culture. We provide the first recorded acoustic signals captured from a collective pressure wave response corresponding to the thermoelastic expansion of cells during X-ray radiation. We demonstrate here that irradiation with 1 Gy of 6 MV X-ray photons causes cultured cells to emit characteristic sound waves during irradiation and propose that this acoustic signal might trigger, or contribute to the triggering, of the bystander response in neighboring cells.

Materials and methods

Cell culture and irradiation

Human leukemia cells (HL-60) and breast cancer cell line (MCF-7) were cultured in RPMI and DMEM media (Sigma-Aldrich, St. Louis, MO), respectively, in the presence of 10% (v/v) heat-inactivated fetal calf serum (Thermo Fisher Scientific, Waltham, MA), 50 U/mL penicillin, and 50 µg/mL streptomycin (Sigma). Cells were seeded on a gelatin matrix in 40-mm petri dishes (Sigma-Aldrich) and incubated at 37°C in 95% ambient air and 5% (v/v) CO2. HL-60 and MCF-7 cells seeded at 1.5 × 10⁶ cells per 4 cm diameter petri dish and exposed 24 h later to 1 Gy of 6 MV X-ray photons generated by a Varian Clinac 2100 at 0.37 Gy/min. At exposure adherent cells were 80% confluent and viability as measured by trypan blue exclusion 95% and 90% for MCF-7 and HL-60 respectively. Both cell types were irradiated as suspensions for comparability, MCF-7 cells were removed from substrate by trypsinization followed by inactivation of trypsin by adding 10% v/v FCS and irradiating immediately.

Bio-acoustic signal recording

Acoustic emissions were measured using a custom large-bandwidth ultrasound transducer with a central frequency of 1 MHz placed in the center of a 40-mm diameter petri dish containing the media and homogeneously distributed cells (Figure 1). The petri dish was placed on the table at the center of the beam, using the visible light cross hairs, at a distance of 100 cm from the source (see dosimetry specification in Supplementary Information file 1). Controls were carried out with exposed and non-exposed (sham irradiated) petri dishes with and without gelatin coating in RPMI, without cells present. For sham irradiations cells were moved to the beam target position for the same amount of time as experimental dishes but the beam not activated. In exposed and controls the petri dish was located precisely in the middle of the field using the optical cross hairs. The field was shown to be effectively homogeneous (see Supplementary file 1). The transducer was connected to a low-noise pre-amplifier with approximately 60 dB gain and recorded with a PicoScope oscilloscope at sample rate 9.766 MS/s with 102.4 nanosecond sample interval. Signals were read out before, during and after dose delivery using cell-free culture media alone as control.

Statistical analysis

The bio-acoustic peaks observed in the recorded sound signals were analyzed using Matlab® software. The average temporal ‘width’ and acoustic power ‘height’ for each sound peak was calculated for the total peaks observed during a set period of time of one second and were carried out using n = 5 biological replicates. The number of peaks per second over a sound pressure voltage over a threshold of −3.5 V were determined for same n = 5 replicates. The significance of the maximum amplitude values of acoustic peaks was calculated for the five replicates per experimental condition with a total of 250 peaks per condition including 50 peaks per replicate. Significant differences between groups were determined using a two-tailed Mann–Whitney U test. A value of p < .05 was deemed statistically significant.

Results

Bio-acoustic signals induced by X-ray radiation

Sound wave signals were recorded before, during and after X-ray radiation in order to experimentally verify the bio-acoustic wave emission generated from a cell-cultured population. Controls were carried out with irradiated and sham irradiated petri dishes without cells, in the presence or absence of gelatin (Supplementary Figure 3). We show that the signal generated by controls is similar in magnitude and frequency distribution irrespective of whether petri dishes were directly irradiated or sham irradiated. The position of the transducer in the irradiated controls was identical to that in experimental determinations, and we conclude that the signal we see when cells are irradiated is due to cell emission and not an artifact of irradiation of the transducer. The recording of the raw temporal acoustic signal for non-adherent HL-60 cells and respective media control RPMI is represented in Figure 2 and for MCF-7 cells in Figure 3.

No sound peaks are observed for the control media samples while sharp sound pressure peaks are recorded for MCF-7 and HL-60 samples which represent the collective pressure response of the cell population in culture. Raw data can be found in Supplementary Table 1 and results of statistical analysis for acoustic wave generation in Supplementary Table 2 and Supplementary Figure 4.

Different dose rates between 0.1 and 0.4 Gy/min at 1 Gy isodose were investigated and showed a dependency of
amplitude on dose rate but not overall dose delivered (Supplementary Figure 4 and Supplementary Tables 1 and 2). The amplitude of the signal from both cell types was dependent on the concentration of cells irradiated (Figures 2 and 3 and Supplementary Figure 4). The only differences between treatment groups that failed to reach a significance of $p < .05$ were between 0.2 and 0.1 Gy/min irradiation of MCF7 cells ($p = .221$) and 0.3 and 0.2 Gy/min for MCF7 ($p = .058$). We demonstrate that during the period of dose delivery, the non-adherent HL-60 cells generated spikes of a sharp acoustic signal of average temporal width of 20 µs at an average rate interval of 357 Hz between each spike. Adherent MCF-7 cells generate spikes of a sharp acoustic signal of average temporal width of 18 µs at an average rate interval of 344 Hz between each spike. The transient thermoelastic expansion of cells during X-ray irradiation generates collective pressure waves that alternate between pressurized and de-pressured phases with observed resting interval before each peak elicited. The sharp pressure spikes start with a pressurization period and followed by de-pressurization period.

The acoustic pressure peak analysis is based on the temporal width (in µs) and sound power amplitude by electroacoustic signal height (in mV). The frequency spectrum

Figure 1. Combined X-ray radiation stimuli with sound recording setup. Schematic experimental setup showing amplified sound recording system combined with X-ray irradiated cultured cells exposed to an X-ray field of 10 x 10 cm. The petri dish and the piezoelectric transducer were placed in the center of the field using the visible light cross hairs 1000 mm from the source.

Figure 2. HL60 concentration dependence on acoustic signal. Raw temporal acoustic signals for adherent HL60 (pink) cells sample for concentration of (B) $0.1 \times 10^6$ cells/mL; (C) $0.5 \times 10^6$ cells/mL; and (D) $1.2 \times 10^6$ cells/mL. Respective media control RPMI without any cells (A) in black and the respective frequency spectrum map of processed acoustic signals for low frequency [0–1 MHz] and for high frequency [3.5–5 MHz] domains as a function of irradiation time.
map of processed acoustic signals for lower range of frequency [0–1 MHz] and for high frequency [3.5–5 MHz] domains as a function of irradiation time shows that the sharp spikes observed correspond to a lower frequency range from $10^{10}/C_0^2$ Hz to 750 kHz and weaker ultrasound signals at higher frequencies observed between 3.5 and 4.8 MHz. The signal does not continue detectably after the end of irradiation, indicating efficient relaxation processes.

**The acoustic signal is dependent on cell viability**

In order to ascertain if the acoustic emissions are dependent on intact living cells we compared irradiation of the same numbers of live and dead cells. The resulting frequency/amplitude profiles are shown in Figure 4.

The signal from irradiated dead cells (Figure 4(A); MCF7, Figure 4(B); HL60) is similar in frequency distribution but different in amplitude to living cells (Figure 4(C); MCF7 and Figure 4(D); HL60) at the same concentration. Supplementary Figure 4 shows significant changes in peak maximum amplitude between irradiated live and dead cells, and comparison in the same experiment between HL60 and MCF7 dead cells ($p = 9.97 \times 10^{-11}$), effectively indistinguishable, suggesting that dead cells are much more similar to each other than live cells of different types (Supplementary Table 2).

**Discussion**

It has previously been shown that X-ray photons elicit an acoustic signal from whole tissues; a property that has been exploited in the development of X-ray-induced acoustic emission tomography (Samant et al. 2020), but these emissions are part of the bulk properties of tissues and the source of emission may be extracellular matrices, water, and tissue fluids.

We demonstrate here for the first time a characteristic radiation-induced acoustic emission (RIAE) from cells in culture. The acoustic signals we record are complex, and differ between the two cell types under investigation. We are unable to determine the reasons for differences between the emissions from HL-60 and MCF 7 cells but the physical properties of the cells may be the source. The difference in RIAE pattern from each cell type is likely to be due to the shape, size and elastic properties of the cells. MCF-7 are adherent cells in a monolayer, and HL-60 non-adherent though settled onto the substrate in this experiment. MCF-7 cells have an average interphase size of 15-17 $\mu$m, while HL-60 cells are smaller at 12.4 ± 1 $\mu$m, and they differ in elastic modulus with HL-60 $E_a = 0.53$ kPa and MCF-7 $E_a = 2.1$ kPa which is a measure of cellular deformability (Rosenbluth et al. 2006). The elasticity and deformability of cells will in principle affect their ability to initiate and respond to an acoustic signal and may affect the nature of the collective pressure wave elicited from each cell type (Nyberg et al. 2017). It is of note that the cell status affects deformability or elasticity, and dead, dying, or challenged cells behave differently to normal cells (Otto et al. 2015), suggesting that although the generation of RIAE might be a non-energy dependent process it may still depend on the structure and state of the living cell.

The observation that emissions cease simultaneously with the cessation of irradiation is consistent with fast physical relaxation processes, and not, for example, the induction of a persistent metabolic energy-dependent acoustic signaling from cells. We show that dead cells also emit an acoustic signal, showing that this is a physical property of cells and not due to some active signal-generating mechanism.
However, the amplitude of the signal from live and dead cells differs significantly, suggesting that there may be some metabolic energy dependent process involved, or a cellular integrity dependent mechanism, influencing this difference, for example the arrangement of the cytoskeleton, or the integrity of physical intercellular connections.

The original motivation for these experiments was the hypothesis that a physical signal, such as sound might explain some of the bystander phenomena observed in cells and between organisms. We have not at this stage attempted to demonstrate a direct link between these signals and RIBE, but we can rule out this acoustic mechanism at least for the inter-organism bystander processes due to the temporal co-dependence of irradiation and RIAE (Mothersill et al. 2012). However, it is still possible that it may play a role within tissues and it may also be associated with, or mediate the release of, other proposed signaling factors, such as exosomes. Data already exist linking acoustic stimulation with calcium dependent exosome release from cancer cells in culture (Ambattu et al. 2020). If RIAE contributes to the bystander effect there must be a sensing mechanism in non-irradiated cells. There are many examples of cellular mechanoreceptors which might be involved in sensing and transducing acoustic signals, discussed in Matarèse et al. (2022). For example, in some mammalian cell types, such as otic hair cells, signal transduction involves ion (Na\(^+\), K\(^+\), and Ca\(^{2+}\)) influx and signaling through the action of the MET mechanoreceptor which detects mechanical stretching and deformation of the hair cell membrane (Fettiplace 2017). We consider that it is of significance that calcium influx is the first measurable event in cells receiving a radiation-induced bystander signal (Lyng et al. 2000).

**Conclusion**

We demonstrate that irradiation of two different cancer cell lines results in the emission of characteristic acoustic signals (Radiation Induced Acoustic Emission [RIAE]). The amplitude of the emission is dependent on cells being alive, but we cannot differentiate between the involvement of metabolic energy-dependent processes and the different physical properties of living and dead cells such as elasticity, which are provided by an intact cytoskeleton or cell membrane. The phenomenon shows dependence on dose rate and can be seen above 0.2 Gy/min. We have not, in these preliminary experiments, attempted to directly and mechanistically link the bystander effect with local acoustic emission, but further work is in progress to establish the relationship between sonic emission and sensing, and the bystander response in vitro. We conclude that irradiation of cultured cells results in a characteristic sonic emission and that this phenomenon needs now to be taken into account when developing hypotheses about the effects of radiation on biota.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.
Data deposition

Data are deposited in STOREDB DOI:10.20348/STOREDB/1169. Individual files can be accessed on: STOREDB:DATASET1245 Raw Picoscope data [DOI:10.20348/STOREDB/1169/1245].

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Data availability statement

Raw acoustic data and measurements of extracellular Lactate dehydrogenase and mitochondrial membrane depolarization are available from the STORE database.

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