Chemical induction of the bystander effect in normal human lymphoblastoid cells

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1. Introduction

Cells are constantly exposed to low levels of DNA damaging agents such as chemicals and radiation. The long-standing belief is that cells must be directly “hit” by an agent for damage to occur. As early as the 1940s there were reports that damage to DNA can be brought about by ionizing radiation that not only targets the cell (or tissue), but also targets the surrounding medium [1, 2]. In spite of considerable evidence is now available that challenges this phenomenon. The term “radiation-induced bystander effect” refers to the response seen in cells that have not been directly exposed to radiation. This phenomenon was initially described by Kotval and Gray [3] who showed α-particles passing close to but not through the chromatin threads were capable of producing chromosomal breaks and exchanges. However, most of our current knowledge is from experiments performed in the past decade. Nagasawa and Little [4] first demonstrated an increase in the induction of sister-chromatid exchanges in cells that were not directly exposed to α-particles but were in the vicinity of directly exposed cells. Extensive work has now shown that radiation-induced bystander effects can be divided into two broad categories: gap-junction mediated which requires cell-to-cell contact [5–7], and secretion of extracellular soluble factors into the culture medium [8–14].

Radiation-induced bystander effects have been observed following exposure to α-particles, X-rays, γ-rays and radioisotopes that emit a variety of disintegration products. Several studies have demonstrated that γ-radiation can induce bystander effects when media from irradiated cells is transferred to unexposed cells, resulting in reduced clonogenic survival [8, 15], increased mitochondrial mass [16] and apoptosis [9, 11, 17]. Increases in the amount and the rate of induction of replication protein-A have been observed in bystander cells compared to directly irradiated cells [18]. Replication protein-A is an important component in DNA repair and is involved in DNA replication and recombination. An increase in the number of micronuclei [19], induction of p53Waf1 and γ-H2AX and apoptosis have been observed in unirradiated bystander cells that were adjacent to cells exposed to γ irradiation [20]. Following exposure to α-particles, increases in chromosomal aberrations [21], sister chromatid exchanges [4, 22], micronuclei [19, 23, 24], DNA double strand breaks [25], mutations [26] and decreased levels of TP53 and CDKN1A [27], as well as changes in gene expression [28, 29] have all been observed in bystander cells. Bystander effects induced by the non-uniform distribution of 125I [30] and 3H [31, 32] in a three-dimensional tissue culture model showed an increase in cell killing in unlabeled cells.

Unlike the work using ionizing radiation, most studies evaluating the effects of chemicals upon bystander responses have been performed in vivo on modified tumors (transfected with α-particles, increases in chromosomal aberrations [21], sister chromatid exchanges [4, 22], micronuclei [19, 23, 24], DNA double strand breaks [25], mutations [26] and decreased levels of TP53 and CDKN1A [27], as well as changes in gene expression [28, 29] have all been observed in bystander cells. Bystander effects induced by the non-uniform distribution of 125I [30] and 3H [31, 32] in a three-dimensional tissue culture model showed an increase in cell killing in unlabeled cells.

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cytokine or suicide gene) to promote inhibitory effects on neighboring unmodified tumors; these observations have also been called the bystander effect [33–35]. Few studies have focused on the ability of chemicals to induce a bystander effect. Recently, the ability of the chemotherapeutic DNA-alkylating agent, chloroethylnitrosourea to induce the bystander effect through soluble factors in primary melanomas was demonstrated [36]. The progeny of cells exposed to mitomycin C (MMC) induced genomic instability in unexposed neighboring cells [37]. However, induction of the bystander effect by chemicals remains poorly characterized.

Here we evaluated the ability of two chemical DNA damaging agents, MMC and phleomycin (PHL), as well as ionizing radiation, to induce a bystander effect through cellular secretion of media soluble factors. MMC is a bifunctional alkylating agent that forms DNA cross-links. PHL is a radiomimetic chemical that produces hydroxyl radicals that result in DNA double strand breaks, and ionizing radiation damages DNA through the direct absorption of energy and indirectly through the accumulation of reactive oxygen species.

The micronucleus assay has been used routinely for decades to evaluate exposure risks to humans by various chemicals as well as ionizing radiation, nutrient deficiency and defects in DNA repair genes [38], and is a standard technique used in genetic toxicology studies [39]. In this study we used micronuclei as an endpoint and demonstrate that the bystander effect can be induced by chemicals, not just ionizing radiation, suggesting that this phenomenon may comprise part of a general cellular stress response.

2. Materials and methods

2.1. Cell culture

Two normal Epstein Barr Virus-transformed human B-lymphoblastoid cell lines (GM15036 and GM15510) were obtained from the Coriell Cell Repository. The cells were grown in suspension in RPMI 1640 medium (GIBCO, Paisley, UK), supplemented with 15% fetal bovine serum (Hyclone, Logan, Utah), penicillin–streptomycin (100 units/ml penicillin G sodium, 100 μg/ml streptomycin in 0.85% saline) (GIBCO, Paisley, UK), 2.5 μg/ml Hyn× four subsequent washes, and 2 mM L-glutamine (GIBCO, Paisley, UK). Cells were sub-cultured by seeding at a concentration of 1.0 × 10^5 cells/ml and were grown in a fully humidified incubator with 5% CO2 at 37°C in T25 culture flasks (Corning, NY).

2.2. Irradiation

Upon reaching a density of 2.0 × 10^5 cells/ml, the 10 ml cultures were acutely exposed to 0 (control), 0.5, 1, 2, 3, and 4 Gy X-rays at a dose rate of approximately 1.8 Gy per minute using a Pantak HF320 X-ray source (Pantak, CT, USA). The cells were then returned to the incubator. Four hours after radiation, the cells were treated with 2 μM hydroxyurea, and 2 mM L-glutamine (GIBCO, Paisley, UK). Cells were then cultured by seeding at a concentration of 1.0 × 10^5 cells/ml and were grown in a fully humidified incubator with 5% CO2 at 37°C in T25 culture flasks (Corning, NY).

2.3. Chemical treatment

Upon reaching a density of 2.0 × 10^5 cells/ml, 10 ml cultures in T-25 flasks were exposed to 0 (control), 50, 100, 200 and 300 ng/ml (final concentration) MMC (Sigma–Aldrich, CAS No. 50-67-7) or 0 (control), 50, 100, 200 and 300 μg/ml (final concentration) PHL (Sigma–Aldrich, CAS No. 11066-33-0). One hour later the cells were washed three times in 10 ml of fresh media to remove residual chemical and then incubated in fresh media for 4 h. The cells were then treated with 6 μg/ml Cytochalasin B (Sigma–Aldrich) and were harvested 28 h later.

2.4. Media transfer

The flasks designated for providing the conditioned medium contained 2.0 × 10^5 cells/ml in 10 ml cultures in T-25 flasks, and were exposed to a range of radiation doses or concentrations of MMC or PHL. For MMC, the cultures were exposed to 0, 50, 100, 200 or 300 ng/ml (final concentration). For PHL, the cells received 0, 50, 100, 200 or 300 μg/ml (final concentration), and for irradiation the cells received 0, 0.5, 1, 2, 3 or 4 Gy. Media from unexposed cells was used for controls. In the case of the chemical treatments, the cells were washed three times in fresh media 1 h after the initial treatment and were then incubated in fresh media for an additional 4 h. The radiated cells were left undisturbed until the media transfer was performed. Four hours after the radiation exposure (or 4 h after washing off the residual chemical), the media transfer was performed by centrifuging the cultures in 15 ml conical tubes at 300 × g for 5 min. The supernatants were then collected and passed through a 0.22 μm filter (Corning, NY) to ensure that no cells were present in the media. This filtrate was considered to be conditioned medium and was used immediately to grow unexposed recipient cells. The original culture media was aspirated from the recipient cells after centrifugation and was replaced with the filtered conditioned media. The recipient cells were grown in the conditioned media, in the presence of 6 μg/ml Cytochalasin B for 28 h and then harvested.

2.5. Micronucleus assay

The cells were collected by centrifugation for 5 min at 300 × g and each pellet was resuspended in 1× phosphate buffered saline (PBS, Fisher Scientific, Pittsburgh, PA). The cells were then spun onto clean microscope slides using a cytocentrifuge (StatSpin, Westwood, MA) for 4 min at 1300 RPM. After drying briefly, the cells were fixed in 100% methanol for 15 min and stored at room temperature in slide boxes prior to staining. The cells were stained with Acidine Orange (0.5 mg/ml in 1× PBS) in the dark for 3 min. The excess stain was removed by a 3 min wash in 1× PBS, followed by 2 subsequent washes in distilled water for 3 min each. The cytokinesis-blocked binucleated cells were scored using a fluorescence microscope (Nikon Eclipse E400). Cytotoxicity was evaluated by determining the Nuclear Division Index [40], which is the average number of nuclei per cell. The micronuclei were identified and scored in accordance with recognized criteria [41]. All slides were coded prior to scoring to prevent observer bias. Five hundred binucleated cells per slide were scored by two individuals for a total of 1000 cells per treatment, including a sham-treated control.

2.6. Statistical analyses

Analysis of variance (ANOVA) was performed to evaluate the significance of the chemical and radiation bystander responses. The three genotoxic agents were analyzed individually. The parameters evaluated were dose (for radiation) or concentration (for the two chemicals), condition (i.e., direct effect vs. bystander effect), and cell line, plus all three possible two-way interaction terms and the three-way interaction term, and an intercept term. The analyses were performed using version 6.0.0 of SAS JMP® software, Cary, NC. The 95% confidence intervals were estimated as described [42].

3. Results

3.1. Lack of residual chemical effect

To eliminate the possibility that residual chemicals might be responsible for the effect we observed in the bystander cells, we washed the chemical-exposed cells three times in fresh media 1 h after exposure. Media from the final wash was used to culture unexposed cells. Had there been residual chemical, cells cultured in media from the last wash would exhibit an increase in micronucleus frequencies compared to cells that had not been exposed. However, this is not the case, as the micronucleus data shown in Fig. 1 clearly demonstrate that the three washes successfully eliminated each chemical (p = 0.28 and p = 0.66, for MMC and PHL, respectively). Thus, the increase in micronucleus frequencies observed in the cells cultured in conditioned media was solely due to the factors released by exposed cells.

3.2. Mitomycin C-induced bystander effect

Treatment with MMC generally showed concentration-related increases in the total number of micronuclei (Fig. 2). For both cell lines, the frequency of micronuclei was elevated 2–5 fold at all the concentrations tested compared to the sham-treated controls. The Nuclear Division Index ranged from 1.90 to 1.37 in GM15510 cells and from 1.98 to 1.31 in GM15036 cells exposed from 0 (control) to 300 ng/ml MMC, respectively. These results indicate that the cells cultured with the highest concentrations that caused damage without excessive cell cycle delay or cell killing, either of which would have resulted in an insufficient number of binucleated cells to complete the analyses.

Cells treated with conditioned media generally showed a concentration-responsive increase in micronuclei, yielding approximately a 1.5–3 fold increase compared to sham-treated controls in both cell lines (Fig. 2). In GM15036 cells there was a slight decrease in the number of micronuclei in cells receiving conditioned media.
Fig. 1. Micronuclei induction in human B-lymphoblastoid cells cultured in media obtained from the third wash that was performed to eliminate any residual chemical. (A) MMC and (B) PHL exposed cells were washed three times with fresh media 1 h following the exposure. The media from the last wash was used to culture unexposed cells and cells were harvested 28 h after treatment or after receiving conditioned media.

from 300 ng/ml treated cells compared to the next lowest concentration.

The data were analyzed using ANOVA (Table 1). MMC concentration, condition (direct or bystander) and the concentration-condition interaction were found to be highly significant. No significant difference was seen between the cell lines. MMC caused concentration-responsive increases in micronuclei in both cell lines, for cells that were directly exposed and for those cultured in conditioned media. For cells that were directly exposed to MMC, the slopes of the regression lines were $y = 45.3 \pm 0.98 \ (r^2 = 0.91)$ and $y = 42.3 \pm 0.089 \ (r^2 = 0.97)$ for GM15510 and GM15036 cells, respectively. Similarly, for cells receiving conditioned media, the slopes of the regression lines were $y = 25.6 \pm 0.050 \ (r^2 = 0.81)$ and $y = 26.7 \pm 0.077 \ (r^2 = 0.64)$ for GM15510 and GM15036 cells, respectively.

3.3. Phleomycin-induced bystander effect

Normal human lymphoblastoid cells treated directly with PHL exhibited a 4–6 fold increase in the number of micronuclei (Fig. 3). The lowest concentration (50 μg/ml) of PHL evaluated resulted in a 4.5 fold increase, compared to the sham-treated controls, in the number of micronuclei in both cell lines. Both cell lines showed little additional increase in micronuclei above 100 μg/ml, suggesting a saturation effect, and GM15510 cells exhibited a small decrease in the number of micronuclei at the highest concentration (300 μg/ml) compared to the next lower concentration. The Nuclear Division Index ranged from 1.96 to 1.27 in GM15510 cells and from 1.87 to 1.23 in GM15036 cells exposed to 0 (control) to 300 μg/ml PHL, respectively.

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Fig. 3. Micronuclei induction in human B-lymphoblastoid cells following direct exposure to PHL or transfer of conditioned media. (A) GM15510 and (B) GM15036 cells were either sham treated (0 μg/ml) or exposed to 50, 100, 200 or 300 μg/ml PHL. The bystander effect cells are unexposed cells cultured in conditioned media. All cells were harvested 28 h after treatment or after receiving conditioned media.

Conditioned media induced a 1.5–4 fold increase in micronuclei in GM15510 cells. However, we observed a slight decrease in the number of micronuclei in cells receiving conditioned media from cells exposed to 200 and 300 μg/ml PHL, compared to 100 μg/ml. GM15036 cells exhibited a concentration-responsive 2.5–4 fold increase compared to controls (Fig. 3).

The data were analyzed using ANOVA (Table 1). PHL concentration and condition (direct or bystander) were found to be highly significant; no significant difference was seen between the cell lines. The slopes of the regression lines were found to be $y = 61.8x \pm 0.43$ ($r^2 = 0.70$) and $y = 53.0x \pm 0.23$ ($r^2 = 0.63$) for cells directly exposed to PHL, and $y = 38.7x \pm 0.30$ ($r^2 = 0.64$) and $y = 35.9x \pm 0.087$ ($r^2 = 0.88$) for cells receiving conditioned media in the case of GM15510 and GM15036 cells, respectively. In the case of GM15510, the highest concentration, 300 μg/ml was not used in the regression analysis.

3.4. Radiation-induced bystander effect

Direct acute exposure to ionizing radiation showed a dose-responsive increase in the number of micronuclei in both cell lines (Fig. 4) up to 3 Gy. At the 4 Gy dose, GM15510 cells exhibited signs of toxicity, as evidenced by a decrease in the number of micronuclei. GM15510 cells showed a higher number of micronuclei for the same radiation dose when compared to GM15036 cells. We observed a 7–14 fold increase in micronucleus frequencies in GM15510 cells, whereas GM15036 cells showed a 4–10 fold increase, compared to the 0 Gy controls. The Nuclear Division Index ranged from 1.96 to 1.27 in GM15510 cells and from 2.00 to 1.29 in GM15036 cells exposed to 0 (control) to 4 Gy of radiation, respectively. This shows that the cells were exposed to the highest possible doses of radiation that still allowed enough binucleated cells to be available for scoring.

Cells treated with conditioned media exhibited a dose-responsive 2–8 fold increase in micronucleus frequencies, when compared to the sham-treated controls (Fig. 4). We observed a 3–8 fold increase in micronuclei numbers up to 3 Gy in GM15510 cells. A slight decline in the micronuclei frequencies in cells cultured in conditioned media obtained from 4 Gy exposed cells, compared to 3 Gy was also observed in this cell line. In the case of GM15036 cells, we observed a dose-responsive 2–6 fold increase in the micronuclei frequencies compared to sham-treated controls.

The data were analyzed using ANOVA (Table 1). Radiation dose, condition (direct or bystander) and the dose–condition interaction were found to be highly significant, and the difference between the cell lines was marginally significant. Radiation caused dose-responsive increases in micronuclei in both cell lines, for cells that were directly exposed and for those cultured in conditioned media. For directly irradiated cells, the slopes of the regression lines were $y = 92.4x \pm 18.94$ ($r^2 = 0.95$) and $y = 57.9x \pm 11.67$ ($r^2 = 0.96$) for GM15510 and GM15036 cells, respectively. Similarly, for cells receiving conditioned media, the slopes of the regression lines were $y = 37.1x \pm 10.44$ ($r^2 = 0.96$) and $y = 28.11x \pm 1.541$ ($r^2 = 0.99$) for GM15510 and GM15036 cells, respectively. The 4 Gy dose was not used in the regression analysis in the case of GM15510 cells.

4. Discussion

Most research on the bystander effect has focused on ionizing radiation and only a few studies have evaluated alkylating agents.
To our knowledge the work described here is the first to evaluate chemically induced bystander effects in normal fully differentiated, human cells and the first to demonstrate the ability of genotoxic chemicals such as MMC and PHL to induce a bystander effect in the same context as ionizing radiation. Our findings indicate that media conditioned by cells cultured in MMC induced a 1.5–3 fold increase in micronuclei, and that media conditioned by cells cultured in PHL induced a 1.5–4 fold increase, and conditioned media from irradiated cells induced a 2–8 fold increase in micronuclei compared to sham-treated controls. Both chemicals as well as radiation induced bystander effects at all the doses and concentrations evaluated. Even at the lowest levels of exposure a minimum of a 50% increase in micronuclei was observed.

There have been two previous studies on chemical-induced bystander effects. One used primary cultures of mouse tumor cells [36] and it is now widely accepted that tumor cells exhibit genomic instability [43]. The other study used mouse embryonic stem cells which exhibit genomic instability following exposure to MMC [37]. It is thus difficult to determine conclusively whether the effect observed in the naïve bystander cells is due to factors secreted by the chromosomally unstable cells or whether the observed effect is truly due to secreted bystander factors. In this study, we used normal human lymphoblastoid cells, which do not exhibit cell-to-cell contacts and thus are appropriate for evaluating bystander effects brought about by media transfer (i.e. secreted soluble factors). Since we evaluated the bystander effect 4h after the initial exposure in normal cells, i.e. a short time after exposure, we can rule out the possibility that factors secreted due to genomic instability (a long-term effect) rather than the bystander effect are responsible for the response observed in the unexposed cells.

PHL, like radiation, has been known to induce genomic instability [44]. DNA lesions induced by MMC have been found to persist for up to 24 h and only 50% were repaired 32 h following exposure [45]. MMC has been used in clinical chemotherapy since the 1960s for up to 24 h and only 50% were repaired 32 h following exposure [46]. DNA lesions induced by MMC have been found to persist for up to 24 h and only 50% were repaired 32 h following exposure [46]. However, recently it was demonstrated that mouse embryonic stem cells exposed to MMC were able to induce homologous recombination in unexposed cells several generations after the exposure and these bystander cells in turn were able to propagate the damage to unexposed neighboring cells [37]. Demidem et al. [36] demonstrated both in vivo and in vitro the ability of chloroethylnitrosurea to induce solid tumors to secrete soluble bystander factors. These soluble factors may either protect the bystander cells from death or help them to resist subsequent exposures to the agent. Alternatively, the factors might exhibit a cytotoxicity effect, resulting in cell death.

The occurrence of radiation-induced bystander effects led to a paradigm shift in the dogma of radiobiology. While the ability of ionizing radiation to induce the secretion of media soluble factors has been well documented [8–14], there is very little literature on the ability of other DNA damaging agents to do the same. A number of endpoints have been used to study radiation-induced bystander effects, many of which are indicators of DNA damage, such as sister-chromatid exchanges [4,22,47–49], micronuclei [13,19,23,24,50–52], chromosomal aberrations [53]. DNA double-strand breaks, γ-H2AX levels [7,13,25,54] and mutations [26]. These endpoints, although considered to be markers of ionizing radiation, are more representative of DNA damage which can be induced by a number of different agents, including chemicals. It is therefore more likely that the DNA damage brought about by the bystander effect is induced not only by ionizing radiation but that any genotoxic agent, whether chemical or physical, can induce a bystander effect.

In recent studies, serotonin, a molecule used to signal stress, was shown to modulate the production of bystander signals by irradiated cells [55]. Up-regulation in the levels of stress-inducible proteins p21WAF1 and p53 was observed in a greater number of cells than were traversed by the α-particles used to trigger the response [6,56]. The MAP kinase pathway has also been shown to be activated in the radiation-induced bystander effect [24,28,56]. This serves as convincing evidence for the hypothesis that the bystander effects as described here are not unique to radiation, but can be induced by a variety of DNA damage causing and stress-inducing agents.

In the present work we show that two genotoxic chemicals, differing in their modes of action, can induce bystander effects in normal lymphoblastoid cells. The chemically induced bystander effect, as witnessed by the increase in the total number of micronuclei in the bystander cells, cannot be due to a residual chemical effect. When we used the media from the last wash to culture unexposed cells, the total number of micronuclei in these cells was comparable to the sham-treated controls, ruling out the possibility that a residual chemical effect was responsible for the increase in micronucleus frequencies.

Taken together, these results expand our current understanding of the bystander effect. It is apparent from the results presented here that chemicals are also capable of inducing bystander effects through media secreted factors, a phenomenon that was previously thought to be limited to ionizing radiation. Evaluation of other stress-inducing agents might provide further insights into the mechanisms of these indirect effects.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References


