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Original Contribution

Induction of bystander effects by UVA, UVB, and UVC radiation in human fibroblasts and the implication of reactive oxygen species

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ABSTRACT

Radiation-induced bystander effects are various types of responses displayed by nonirradiated cells induced by signals transmitted from neighboring irradiated cells. This phenomenon has been well studied after ionizing radiation, but data on bystander effects after UV radiation are limited and so far have been reported mainly after UVA and UVB radiation. The studies described here were aimed at comparing the responses of human dermal fibroblasts exposed directly to UV (A, B, or C wavelength range) and searching for bystander effects induced in unexposed cells using a transwell co-incubation system. Cell survival and apoptosis were used as a measure of radiation effects. Additionally, induction of senescence in UV-exposed and bystander cells was evaluated. Reactive oxygen species (ROS), superoxide radical anions, and nitric oxide inside the cells and secretion of interleukins 6 and 8 (IL-6 and IL-8) into the medium were assayed and evaluated as potential mediators of bystander effects. All three regions of ultraviolet radiation induced bystander effects in unexposed cells, as shown by a diminution of survival and an increase in apoptosis, but the pattern of response to direct exposure and the bystander effects differed depending on the UV spectrum. Although UVA and UVB were more effective than UVC in generation of apoptosis in bystander cells, UVC induced senescence both in irradiated cells and in neighbors. The level of cellular ROS increased significantly shortly after UVA and UVB exposure, suggesting that the bystander effects may be mediated by ROS generated in cells by UV radiation. Interestingly, UVC was more effective at generation of ROS in bystanders than in directly exposed cells and induced a high yield of superoxide in exposed and bystander cells, which, however, was only weakly associated with impairment of mitochondrial membrane potential. Increasing concentration of IL-6 but not IL-8 after exposure to each of the three bands of UV points to its role as a mediator in the bystander effect. Nitric oxide appeared to play a minor role as a mediator of bystander effects in our experiments. The results demonstrating an increase in intracellular oxidation, not only in directly UV-exposed but also in neighboring cells, and generation of proinflammatory cytokines, processes entailing cell damage (decreased viability, apoptosis, senescence), suggest that all bands of UV radiation carry a potential hazard for human health, not only due to direct mechanisms, but also due to bystander effects.

Radiation-induced bystander effects, which appear in nontargeted cells mainly as cell-damaging events (decreased viability, reduction of clonogenic survival, induction of apoptosis, and cytogenetic damage), are well known phenomena in the case of ionizing radiation [1–4], but knowledge of bystander effects after ultraviolet radiation (UVR) is limited. UVR comprises three different wavelength bands, long-wave UVA (320–400 nm), middle-wave UVB (290–320 nm), and short-wave UVC (200–290 nm) [5,6]. The main source of UVR in the environment is solar radiation, of which about 95% is UVA and ~5% is UVB; UVC is almost completely absorbed in the upper part of the stratosphere [5] unless it traverses an ozone hole in this layer. Bystander effects [7–9] and related genomic instability [10,11] have been reported after UVA and UVB radiation, but very limited data are available on UVC-induced bystander effects, probably because of less interest because this wavelength does not reach the earth. The short-wave radiations (UVB under 300 nm and UVC) are especially dangerous for cells because their bands coincide with the absorption spectra of DNA, RNA, and proteins and they can damage DNA by forming cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs), which can lead directly or indirectly to DNA strand breaks [12–14] and possibly to mutation and neoplastic transformation [15].

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UVR is responsible for the induction and promotion of basal and squamous cell skin cancer [16] and is also an important etiological factor in malignant melanoma [17,18]. Data about the possible significance of bystander effects in UV carcinogenesis come exclusively from in vitro studies. Human keratinocytes whose precursor generations were exposed to UVA showed a reduction of clonogenic cell survival [9] and persistent genomic instability [7]. A reduction of clonogenic cell survival, genomic instability, and delayed mutation has been also observed in bystander Chinese hamster fibroblasts after exposure to UVA and UVB [10,11]. Furthermore, apoptosis was observed in bystander human keratinocytes after both UVA and UVB exposure [8], although in another study [19] no bystander effect was found after UVB radiation.

Bystander effects induced by ionizing radiation [20–22] as well as ultraviolet radiation [7,11,23] are reduced in the presence of antioxidants and may be linked to oxidative stress. Each region of the ultraviolet spectrum induced the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in calf thymus DNA and in HeLa cells in a fluence-dependent manner, with singlet oxygen ($^1O_2$) playing the predominant role [24]. UVC induced DNA double-strand breaks measured by γ-H2AX and 53BP1 foci formation in bystander human fibroblasts more effectively than in irradiated cells, and this effect was mediated by reactive oxygen species (ROS) [23]. Here we report that all three UV wavelength bands, UVA, UVB, and UVC, induce bystander effects in human dermal fibroblasts with a pattern of responses that differs for each. Studies of the levels of ROS and reactive nitrogen species (RNS) and changes in mitochondrial membrane potential suggest that ROS are implicated in this induction. It is known that ROS induced by UV radiation can damage DNA and lead to various skin diseases and carcinogenesis [reviewed 25]. The high production of interleukin 6 (IL-6), a mediator of inflammation, points to its possible role in the induction of UV radiation-induced bystander effects. Although further studies are required to gain knowledge of the detailed nature of the mediators and their interactions, the present results suggest that all bands of UVR carry a potential hazard for human health not only due to direct mechanisms, but also due to bystander effects.

Materials and methods

Cells and experimental procedure

Neonatal human dermal fibroblasts (NHDF-Neo, Lonza, Poland) in early (10–13) passages were grown in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham medium (Sigma–Aldrich), supplemented with 12% fetal bovine serum (PAA, Immuniq, Poland) and 80 μg/ml gentamycin (Krka, Poland). Irradiated and nonirradiated cells were co-incubated in six-well dishes with an insert separating the two cell populations by a 0.4-μm-pore membrane (BD Immunogen) to allow diffusion of medium components between them, as described previously [26]. About 20 h before irradiation cells were seeded into wells (1×10^5 cells/well in 2 ml medium) and those not to be irradiated (bystander cells) were seeded on inserts. Before irradiation the medium was removed and the cells in wells were irradiated (covers opened) at room temperature (21 °C) with various doses of UVA (365 nm), UVB (302 nm), or UVC (254 nm) generated by UV crosslinkers (CL-1000 models, UVP, Upland, CA, USA). We used doses of 5–20 kJ/m^2 (UVA), 2–10 kJ/m^2 (UVB), and 10–200 J/m^2 (UVC). Immediately after irradiation, 2 ml of fresh medium was added to the wells, and then the inserts, also with medium changed, were inserted and the cells were cocultured in a CO_2 incubator (standard conditions: 5% CO_2, 80% humidity, 37 °C) for the desired period.

Cell survival assays

The proportion of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt (MTS) reduction assay (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, Promega). MTS is bioreduced by the dehydrogenase enzymes present in live, metabolically active cells to the colored product formazan. The quantity of formazan measured by the absorbance is directly proportional to the number of surviving cells. The manufacturer's protocol was adapted for our experimental system. Briefly, cells were harvested separately from wells and inserts by trypsinization, spun down, washed in phosphate-buffered saline (PBS), and loaded with MTS reagent. The suspensions were transferred to 96-well plates and incubated for 60 min in a humidified CO_2 incubator, and absorbance was measured at 490 nm using a universal plate reader (Epoch, Biotek Instruments). Survival of control, irradiated, and bystander cells is presented as mean absorbance ± SD from three independent experiments.

Apoptosis and necrosis assays

Apoptosis and necrosis were assessed by flow cytometry using the Dead Cell Apoptosis Kit with annexin V–FITC and propidium iodide (PI; Invitrogen). Annexin V is bound to phosphatidylserine, which is translocated from the inner to the external membrane layer at an early stage of apoptosis [27]. Cells were exposed to 20 kJ/m^2 UVA, 10 kJ/m^2 UVB, or 200 J/m^2 UVC and co-incubated with unexposed cells for the desired time. Cells were harvested separately from wells and inserts, spun down, washed with PBS, suspended in staining buffer, and incubated for 15 min with annexin V–FITC according to the manufacturer's protocol. PI, which stains necrotic cells, was added and the distribution of living, apoptotic, and necrotic cells was measured by flow cytometry (BD FACSAria III) using excitation/emission maxima of 494/518 nm for annexin V–FITC and 535/617 nm for PI. Ten thousand cells were counted. Results are presented as mean fluorescence intensities ± SD from three independent experiments.

ROS assay

Total cellular ROS were assayed as described elsewhere [26] using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma), which was deacetylated to nonfluorescent DCFH by intracellular esterases and then converted by cellular ROS to oxidized, fluorescent DCF. After co-incubation for 3, 6, 12, or 24 h, irradiated and bystander cells were harvested, suspended in growth medium, and loaded with DCFH-DA (final concentration 30 μM) for 30 min at 37 °C in the dark. After the cells were washed in PBS to remove extracellular dye, suspended in PBS, and incubated for 15 min on ice in the dark, ROS were determined in 10,000 cells by flow cytometry using the FITC configuration with excitation and emission wavelengths of 488 and 530 nm, respectively. Results are expressed as mean fluorescence intensities ± SD from three independent experiments.

Measurement of superoxide radical anions

The MitoSOX red mitochondrial superoxide indicator (Invitrogen), which permeates into live cells and selectively targets mitochondria [28,29], where it is rapidly oxidized by superoxide but not by other ROS or RNS and emits red fluorescence, was used according to the manufacturer's protocol. Cells from wells and inserts were harvested separately, spun down, suspended in medium, loaded with MitoSOX (final concentration 5 μM), and incubated for 20 min at 37 °C. Fluorescence was measured in