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BIOLOGY CONTRIBUTION

RECIPROCAL PARACRINE INTERACTIONS BETWEEN NORMAL HUMAN EPITHELIAL AND MESENCHYMAL CELLS PROTECT CELLULAR DNA FROM RADIATION-INDUCED DAMAGE

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Purpose: To explore whether interactions between normal epithelial and mesenchymal cells can modulate the extent of radiation-induced DNA damage in one or both types of cells.

Methods and Materials: Human primary thyrocytes (PT), diploid fibroblasts BJ, MRC-5, and WI-38, normal human mammary epithelial cells (HMEC), and endothelial human umbilical cord vein endothelial cells (HUV-EC-C), cultured either individually or in co-cultures or after conditioned medium transfer, were irradiated with 0.25 to 5 Gy of γ-rays and assayed for the extent of DNA damage.

Results: The number of γ-H2AX foci in co-cultures of PT and BJ fibroblasts was approximately 25% lower than in individual cultures at 1 Gy in both types of cells. Reciprocal conditioned medium transfer to individual cultures before irradiation resulted in approximately a 35% reduction of the number γ-H2AX foci at 1 Gy in both types of cells, demonstrating the role of paracrine soluble factors. The DNA-protected state of cells was achieved within 15 min after conditioned medium transfer; it was reproducible and reciprocal in several lines of epithelial cells and fibroblasts, fibroblasts, and endothelial cells but not in epithelial and endothelial cells. Unlike normal cells, human epithelial cancer cells failed to establish DNA-protected states in fibroblasts and *vice versa*.

Conclusions: The results imply the existence of a network of reciprocal interactions between normal epithelial and some types of mesenchymal cells mediated by soluble factors that act in a paracrine manner to protect DNA from genotoxic stress.

Radiation, DNA damage, γ-H2AX, Epithelial–mesenchymal cell interaction, Paracrine factors.

INTRODUCTION

Radiation response is complex, and the intricacy further increases when effects are examined at the level of tissue. Developmentally every tissue accommodates different types of cells, for example, those of epithelial and mesenchymal origin in parenchymal organs. Tissue homeostasis is dynamically maintained by reciprocal endocrine and paracrine interactions between epithelium and stroma. However knowledge about the role of epithelial and stromal cell cooperation in radiation response is limited.

Several previous studies have addressed this issue. An increased clonogenicity and radioresistance of human head-and-neck squamous carcinoma cells have been observed when they were co-cultured with human fibroblasts, suggestive of a radioprotective effect of epithelial and stromal cell cooperation (1). Similarly human umbilical vein endothelial cells (HUVEC) co-cultured with U87 glioblastoma cells have been found to acquire resistance to radiation-induced apoptosis (2). In contrast, co-culturing of normal dermal fibroblasts with human breast cancer cell line MCF-7 in three-dimensional collagen gels resulted in radiosensitization of the latter (3). Co-culture of preirradiated autologous fibroblasts increased radiosensitivity of megacolonies of murine cervical squamous carcinoma cells (4) and also endothelial cells radiosensitized oropharyngeal squamous carcinoma cells in collagen gels (5). Together these data imply, although somewhat controversially, that interactions between epithelial and mesenchymal cells can modulate the effects of radiation exposure.

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Of note, the above-mentioned works have examined manifestations of radiation response in the context of interactions of mesenchymal cells with transformed epithelium, *i.e.,* in cancer treatment settings. Even less, if anything, is known about an ability of different types of normal cells to affect each other's capacity to cope with radiation. Damage to normal tissues inevitably occurs during radiation therapy, nuclear medicine procedures, occupationally in some professionals, and during involuntary exposure to the environment or accidents involving radiation. Therefore, understanding cross-talk between different types of normal cells exposed to radiation is important from biologic, medical, and public health viewpoints.

This study set out to investigate the reciprocal influence of different types of normal cells on the extent of radiation-induced DNA damage. More specifically, we used primary human thyrocytes and human mammary epithelial cells (HMEC) as representatives of epithelial tissues. The thyroid and breast are among the most radiation-sensitive organs in humans beings, as seen from the studies of carcinogenic effects of medical exposures, the atomic bombing of Japan (6, 7), and the Chernobyl accident (8–10). Several lines of human diploid fibroblasts and endothelial cells were tested as a cellular component of the stroma. DNA damage after irradiation was evaluated as a number of γ-H2AX–containing foci that, although not equivalent, correlate well with DSBs (11) and are commonly used for quantitative measurements. Cell co-culture and conditioned medium transfer were used to address the mechanisms of intercellular interactions.

METHODS AND MATERIALS

Cell lines

Primary human thyroid cell (PT) cultures were established as described previously (12) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO):F12 (Sigma) (1:2) medium supplemented with 3.3% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Sigma). These cultures contain at least 90% of epithelial cells according to cell morphology. In the course of this work, 11 independent PT cultures were used. Normal human foreskin diploid fibroblasts BJ (ATCC, Manassas, VA) and normal human fetal lung fibroblast lines MRC-5 and WI-38 (JCRB, Japan) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mmol/l sodium pyruvate (Sigma), and 1X nonessential amino acids (Sigma) unless otherwise specified. The BJ, MRC-5, and WI-38 cells were used during passages 14 to 26, 19 to 24, and 16 to 21, respectively. A normal human endothelial cell strain, HUV-EC-C (HSRRB, Japan), was maintained in EGM-2 medium (Cambrex, Walkersville, MD) supplemented with 2% fetal bovine serum and 1% penicillin/streptomycin; cells were used during passages 31 to 35. Normal HMEC (Cambrex) were cultured in HEGM-2 medium (Cambrex) and used during passages 9 to 10. Human anaplastic thyroid carcinoma cell lines ARO and FRO, papillary thyroid carcinoma cell lines NPA and TPC-1, and breast cancer cell line MCF-7 were maintained in RPMI-1640 medium supplemented with 5% FBS and 1% penicillin/streptomycin. All cell cultures in this study were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Individual cell cultures and co-cultures

In monocultures, cells (1×10^5/well) were seeded onto sterilized glass coverslips in the 12-well plates, yielding 100% confluent cultures. In co-cultures, PT and BJ cells were mixed at a ratio of 1:1 and cultured in the medium used for fibroblasts or PT. To discriminate between different types of cells (*i.e.,* PT and BJ) under a fluorescent microscope in co-cultures, BJ cells were prelabeled with 500 μmol/l Mito Tracker Red 580 (Molecular Probes, Invitrogen, Eugene, OR) for 15 min at 37°C. Individually or co-cultured cells were incubated overnight before irradiation or other treatments.

Conditioned medium transfer

Medium was conditioned on confluent cultures of each cell line overnight. The next day recipient cells were washed with phosphate-buffered saline (PBS), and conditioned medium (CM) was added after filtration though a 0.22-μm sterile syringe filter or intact. (Filtration or centrifugation of the conditioned medium at 16,000 *g* for 10 min at room temperature did not significantly change results; data not shown.) After CM transfer, cells were incubated for different periods before irradiation, as described later here.

Irradiation and immunofluorescence

Cells grown on coverslips were exposed to a single acute dose of 0 to 5 Gy of γ-rays from a ^{137}Cs source (PS-3100SB, Pony, Japan) at a dose-rate of 1 Gy/min and incubated at 37°C for 30 min unless otherwise specified. Fixation and immunostaining with the monoclonal anti-phosphorylated histone H2AX (Ser139) antibody (Upstate Biotechnology, Lake Placid, NY), anti-phosphorylated ATM (Ser1981) (Rockland, Gilbertsville, PA), anti-53BP1 (Bethyl Laboratories, Montgomery, TX) or antireplication protein A (RPA) (Oncogene Research Products, San Diego, CA) were done as described (13). After incubation with Alexa Fluor 488-labeled antimouse IgG (Molecular Probes, Eugene, OR) in the presence of 10 μg/ml RNAse A (Qiagen, Hilden, Germany), cover slips were washed with PBS, stained with TO-PRO-3 iodide (Invitrogen) and mounted with GEL/MOUNT medium (Biomeda, Foster City, CA). The images were captured with a LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). γ-H2AX foci in at least 100 nonoverlapping nuclei were automatically counted for each data point using the Count/Size procedure of the Image-Pro Plus version 4.5 software package (Media Cybernetics, Inc., Bethesda, MD). Details of parameter settings are available from the authors upon request.

Medium treatment with lindane, heating, and trypsin

The PT cells and BJ fibroblasts were incubated in individual cultures or in co-cultures for 6 h, after which the medium was supplemented with 50 μmol/l lindane (1,2,3,4,5,6-hexachlorocyclohexane, Wako, Japan) or DMSO as a vehicle (14), and the cultures were incubated for additional 18 h before irradiation. The efficacy of gap junction intercellular communication (GJIC) blockage was controlled using scrape-loading/dye transfer technique (15); data not shown.

For heat inactivation experiments, CM from PT and BJ cultures was incubated at 37°C or 56°C for 30 min and transferred to recipient cells which were then irradiated with 1 Gy of γ-rays 1 h later.

For proteolytic treatment of CM, plain DMEM conditioned overnight on confluent cells was used. In our preliminary experiments we found that both PT and BJ cells tolerate unsupplemented DMEM during 24 h without changes in cell morphology. A 1-ml quantity of conditioned DMEM was incubated at 37 °C for 3 h under mild swirling with 130 to 140 mg of PBS-washed and minicolumn-spun BSA beads (made by coupling the protein to BrCN-activated Sepharose 4B, Amersham Biosciences, Stockholm, Sweden) or