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The impact of ionizing radiation on placental trophoblasts

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ABSTRACT

Introduction: Exposure to low-dose radiation is widespread and attributable to natural sources. However, occupational, medical, accidental, and terrorist-related exposures remain a significant threat. Information on radiation injury to the feto-placental unit is scant and largely observational. We hypothesized that radiation causes trophoblast injury, and alters the expression of injury-related transcripts *in vitro* or *in vivo*, thus affecting fetal growth.

Methods: Primary human trophoblasts (PHTs), BeWo or NCCIT cells were irradiated *in vitro*, and cell number and viability were determined. Pregnant C57Bl/6HNsd mice were externally irradiated on E13.5, and placentas examined on E17.5. RNA expression was analyzed using microarrays and RT-qPCR. The experiments were repeated in the presence of the gramicidin S (GS)-derived nitroxide JP4-039, used to mitigate radiation-induced cell injury.

Results: We found that survival of *in vitro*–irradiated PHT cell was better than that of irradiated BeWo trophoblast cell line or the radiosensitive NCCIT mixed germ cell tumor line. Radiation altered the expression of several trophoblast genes, with a most dramatic effect on CDKN1A (p21, CIP1). Mice exposed to radiation at E13.5 exhibited a 25% reduction in mean weight by E17.5, and a 9% reduction in placental weight, which was associated with relatively small changes in placental gene expression. JP4-039 had a minimal effect on feto-placental growth or on gene expression in irradiated PHT cells or mouse placenta.

Discussion and conclusion: While radiation affects placental trophoblasts, the established placenta is fairly resistant to radiation, and changes in this tissue may not fully account for fetal growth restriction induced by ionizing radiation.

1. Introduction

Exposure to ionizing radiation remains a reality in today's world. Worldwide, the average annual exposure to natural radiation is about 2.4 milli Sievert (mSv) [1]. Occupational exposures are most relevant to people working with nuclear fuel and medical devices, in defense-related functions, and in occupations associated with enhanced exposure to natural sources of radiation. For example, aircrew members are exposed to 5–8 mSv per hour while flying [1]. Medical sources of radiation include diagnostic procedures that expose individuals to low doses (commonly 0.1–10 mSv) and therapeutic exposures, typically 20–60 Gray (Gy), to a targeted tissue [1]. Accidental exposures in nuclear fuel processing plants typically expose workers to 1–20 Gy [1]. These risks may be greatly amplified if "dirty bombs" are deployed by terrorists against civilians [2].

Research into diagnosis, treatment, and prevention of radiation injury in pregnancy is limited by appropriate ethical concerns and by the scarcity of information on mechanisms underlying the effect of ionizing radiation on the developing feto-placental unit. Anecdotal reports or observational studies have generated some information pertaining to gestational age and radiation dose. During the pre-implantation period, as little as 0.3 Gy is lethal to the mouse embryo [3]. In the post-implantation period, the main risks from radiation include embryonic death, congenital anomalies, growth restriction, and neurologic maldevelopment [4]. Exposing mice at

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E14 to 0.3–1.5 Gy of whole body irradiation caused decreased neonatal body length and body weight [5]. Minimal effects on litter size or fetal growth were observed when mice at E7-16 were exposed to low dose radiation, 10–13 mSv per day over 10 days [6]. In humans, data from children exposed to *in utero* radiation after catastrophic events in Hiroshima, Nagasaki, and Chernobyl revealed lower height and weight in adolescence [4,7].

Ionizing radiation damages tissues through diverse mechanisms [8]. A major consequence of radiation is direct and indirect DNA damage. Direct effects include the transfer of kinetic energy from radioactive particles to the DNA backbone, which breaks phosphodiester bonds. Indirect effects include the generation of reactive oxygen species, which cause DNA double-strand breaks and cell-cycle arrest. Other types of injury include p53-dependent and -independent apoptosis [9], mitochondrial damage, loss of regenerative capacity, and premature senescence [8]. NFκB mediates several radiation-stimulated signal transduction pathways, which may explain the degree of radiation-sensitivity of differing cells types [10]. These pathways implicate CDKN1A (also known as p21, CIP1), epidermal growth factor receptors, and the apoptosis-related proteins BAX and BCL2 in radiation injury [11]. Whereas radiation-induced pathways have been interrogated in non-placental cell types, there are no studies of radiation injury to cultured primary human trophoblast (PHT) cells; there has been a single study that included the choriocarcinoma line JEG3 and showed no effect on gene expression of gap junction protein alpha 1 [12].

Methods to scavenge reactive oxygen species have been proposed to mitigate radiation damage. This effect has been attributed, at least in part, to the action of manganese superoxide dismutase (MnSOD, [13]). The nitroxides, which have superoxide dismutase–mimetic activity and inhibit lipid peroxidation [14], constitute one such class of radioprotectors. JP4-039 is a nitroxide linked to a short alkene isostere analog of hemigramicidin S, which allows concentration at the mitochondrial membrane, the site of radiation-induced lipid peroxidation [15]. It has been shown to protect against radiation damage *in vivo* [16,17]. In this study, we tested the hypothesis that ionizing radiation causes injury to PHT cells *in vitro* and to the mouse placenta *in vivo*. We also assessed whether the nitroxide JP4-039 mitigates that damage.

2. Materials and methods

2.1. Cell culture and irradiation in vitro

All studies involving human placental cells were approved by the Institutional Review Board at the University of Pittsburgh. For control, we used the immortalized choriocarcinoma line BeWo (ATCC, Manassas, VA), which captures aspects of trophoblast biology but maintains its undifferentiated state and proliferative capacity [18], and NCCIT cells (ATCC, Manassas, VA), a mixed germ cell tumor line that is particularly radiosensitive [19]. Term PHT cells were isolated and cultured using a modified version of the trypsin–deoxyribonuclease–dispase/Percoll method described by Kliman et al. [20,21]. After 4 h in culture, non-adherent cells and syncytial fragments were removed by washing in phosphate-buffered saline (PBS). All human placental cells were maintained, for 72 h after plating, in Dulbecco's modified Eagle's medium (DMEM, Fisher Scientific, Hampton, NH) containing 10% fetal bovine serum (Fisher Scientific), 20 mmol/l HEPES (pH 7.4, Sigma–Aldrich, St. Louis, MO), and antibiotics at 37 °C in a 21% oxygen/5% carbon dioxide atmosphere. The quality of PHT cells was routinely monitored every 24 h by cell morphology and by ELISA assay of medium human beta chorionic gonadotropin (β-hCG, DRG International, Mountainside, NJ), showing a characteristic increase in medium β-hCG as cytotrophoblasts differentiate into syncytiotrophoblasts [21,22].

The cells were irradiated 24 h after initial plating, defined as time zero. Cells were irradiated at the dosage noted in Results vs. sham, defined as 0 Gy [23], using a Clinac 600C (Varian Medical Systems, Palo Alto, CA) with a 6 MV photon beam and a dose rate of 250 cGy/min. The flasks containing the cells were placed on 1.5 cm of bolus (a tissue equivalent material) since the maximum irradiation depth was 1.5 cm, which corresponded to the plated cell layer. In some of the experiments, irradiated cultured cells were exposed to either JP4-039 (10 μM) in DMSO [24], added to the medium 1 h before radiation, or to DMSO alone. Cells were collected for microarray analysis at 4, 8, and 24 h after irradiation, and for all other analyses at 24 h after exposure to radiation.

Cell numbers (BeWo and NCCIT) were assessed by rinsing the monolayer with PBS, followed by trypsinization, resuspension in DMEM, and counting using a hemocytometer. Total cellular protein concentration was measured with the Pierce BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL). For DNA extraction, PHT cells were lysed in a buffer containing NaCl 0.4 M, Tris 10 mM, EDTA 2 nM, SDS 25% with proteinase K (200 μg/ml). The DNA was precipitated in saturated NaCl and isopropanol, washed, resuspended in Tris, and quantified using a NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE).

2.2. Irradiation of pregnant mice

All experiments using mice were approved by the Institutional Animal Care and Use Committee protocols at the University of Pittsburgh. Female C57Bl/6HNsd adult mice were fed standard laboratory chow. Mice were mated and separated the next morning (termed E0.5). Pregnancy was confirmed by weight change on E12.5. On E13.5, mice were irradiated at 0 Gy (sham) or 4 Gy using a Gamma Cell cesium irradiator (JL Shepherd, San Fernando, CA), with a dose rate of 70 cGy/min. This radiation dose was selected based on our experience with mouse irradiation [25], and because a higher dose increased the risk of fetal or early neonatal death, or severe malformation (data not shown). All mice were unanesthetized at the time of irradiation.

Ten minutes after radiation or sham, half the mice from each group received JP4-039, prepared as previously described [26,27]. JP4-039 was dissolved at a concentration of 3 mg/ml in 10% ethanol, 10% Cremophor EL (BASF SE, Limburgerhof, Germany), and 80% water and administered intravenously at a dose of 10 mg/kg, with control mice receiving solvent alone. The mice were monitored for 4 days, and on E17.5, they were re-weighed and then sacrificed. Fetuses and placentas were procured and immediately weighed. One portion of the placenta was placed in RNAlater (Qiagen, Valencia, CA) overnight at 4 °C, then frozen at −80 °C until RNA extraction. Another portion was snap-frozen in liquid nitrogen. Tissue was also fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, stained with hematoxylin and eosin, and microscopically examined using a Nikon Eclipse 90i microscope equipped with Nikon Elements software (both, Nikon Corporation, Tokyo). The image was then imported into ImageJ, version 1.44k (Rasband, W.S., ImageJ, U. S. NIH, Bethesda, MD), for stereologic analysis. Using the methods outlined by Howard [28], we processed vertical uniform random sections of mouse placenta with the Grid plugin. Systematic random vertical lines, 500,000/μm^2 with random offset, were used to measure the height of the labyrinth and junctional zones.

2.3. RNA extraction and processing for RT-qPCR and microarrays

RNA was extracted from PHT cells using both TRI Reagent (Molecular Research Center, Cincinnati, OH) and the RNeasy Mini Kit (Qiagen) and following the respective manufacturers' instructions. For extraction of mouse placental RNA, each placental specimen was homogenized and RNA extracted using TRI Reagent (Molecular Research Center) as above. RNA samples were incubated with DNase I, using a TURBO DNase Kit (Life Technologies, Grand Island, NY), and RNA quality and quantity were determined using a NanoDrop 1000 spectrophotometer (NanoDrop) as well as visualization on a denaturing agarose gel.

Reverse transcription of 1 μg of total RNA to cDNA was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies), with amplification in a Veriti thermal cycler (Applied Biosystems/Life Technologies Corp). Synthesized cDNA was then diluted 1:5 using nuclease-free water. Quantitative PCR was performed in duplicate. For *in vivo* analysis, ribosomal protein L32 (*Rpl32*) was the internal control, while for *in vitro* analysis, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) was the internal control. Quantitative PCR was performed using a 384-well plate with a total reaction volume of 10 μl that included 3 μl of cDNA, 1 μl of forward primer, 1 μl of reverse primer, and 5 μl of SYBR Green PCR Master Mix (Life Technologies). Quantitative PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Because each placental preparation yielded cultured trophoblasts that were subject to radiation or control, gene expression *in vitro* was performed using the delta delta CT method [29], whereas the delta CT method was used for samples derived from *in vivo* experiments.

We used high-throughput microarray analysis to screen for radiation-induced transcriptional changes in cultured PHT cells or mouse placentas. All samples were first examined with an Agilent High-Resolution C Scanner (Agilent Technologies, Santa Clara, CA) to ensure RNA integrity and quality. For cultured PHTs, we analyzed the RNA using the Agilent SurePrint G3 Human GE 8 × 60K arrays (Agilent Technologies). Mouse placental RNA was analyzed using the MouseWG-6 Expression BeadChip arrays (Illumina, San Diego, CA). Microarray data were analyzed using a moderated t-statistic [30]. We then ranked the $\log_2$ expression ratio (radiation:-sham) for each significantly changed transcript. For the PHT RNA data, which encompassed 3 time points (4 h, 8 h, and 24 h), we ranked transcripts by the maximum $\log_2$ expression ratio over the entire 24 h time course. We then selected a merged subset of the top 1% and bottom 1% of differentially expressed RNA from PHTs and from mouse placentas.