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Radiation-Induced Apoptosis Varies Among Individuals and is Modified by Sex and Age

Mark A. Applebaum^{1,*}, Andrew D. Skol^{2,*}, Elisabeth E. Bond³, Michael Overholtzer⁴, Gareth L. Bond³, and Kenan Onel^{1,5,6}

¹Department of Pediatrics, The University of Chicago, Chicago, Illinois 60637

²Department of Medicine, The University of Chicago, Chicago, Illinois 60637

³Ludwig Institute for Cancer Research, ORCRB, University of Oxford, Oxford, OX3 7DQ, United Kingdom

⁴Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10065

⁵Committee on Cancer Biology, The University of Chicago, Chicago, Illinois 60637

Abstract

Purpose—Although there are considerable data on mechanisms of radiation-induced apoptosis *in vitro* and in animal models, little is known about functional variation in these pathways in humans. We sought to develop a tractable system to evaluate this.

Materials and methods—Peripheral blood mononuclear cells were isolated from 90 healthy volunteers, divided into two aliquots, one irradiated with a 5 Gy dose and the other sham-treated (0 Gy), and assessed for damage-induced apoptosis after 24 hours. To investigate reproducibility, ten individuals spanning the entire radiation-induced apoptotic range were tested three times each, with 3–6 months between replicates.

Results—We observed surprising heterogeneity in apoptosis among individuals, ranging from 21–62%. Biological replicates from a single individual, however, were completely concordant, suggesting the variability observed across individuals is not the result of stochastic or short-term effects. We found significantly higher radiation-induced apoptosis in males than in females (Mean: 41.0% vs. 30.7%; $p < 3.5 \times 10^{-7}$). Moreover, advancing age was associated with decreasing radiation-induced apoptosis in males ($p = 0.01$) but not females ($p = 0.82$).a

Conclusions—Our results provide evidence that the function of cellular pathways crucial for stress-induced apoptosis varies by sex and could decline with age in humans.

⁶Corresponding Author: Kenan Onel, M.D., Ph.D., Section of Hematology/Oncology, Department of Pediatrics, The University of Chicago, 900 East 57th Street, KCB 5140, Chicago, Illinois 60637, Tel: 773-702-4919, Fax: 773-834-1329, konel@uchicago.edu.

*These authors contributed equally to this work

Declaration of Interest

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Introduction

From routine air travel and myriad medical applications to nuclear power plants, ionizing radiation (IR) exposure is nearly ubiquitous in modern society. Despite its many benefits, there are also significant deleterious consequences associated with radiation; it is one of very few complete carcinogens, able to both induce and promote cancer (Williams 2008). Because of its dual capacity for good and harm, there is considerable interest in identifying bioassays that are either prognostic for IR-induced side effects or that can be used to investigate the basis of inter-individual variability in radiation responsiveness.

There have been attempts to model variability in the response to IR. Lymphoblastoid cell lines (LCL), for example, have been used to investigate differences in the function of pathways involved in DNA repair and apoptosis (Veldwijk et al. 2011, Chiba et al. 2012). They have also been used to identify genetic determinants of pathways involved in the cytotoxic response to IR (Shukla and Dolan 2005, Hartford and Dolan 2007, Niu et al. 2010). Recently, peripheral blood mononuclear cells (PBMC) isolated from a small panel of healthy volunteers were also used to identify genes associated with radiation response (Budworth et al. 2012). It remains unclear, however, whether immortalized cell lines are appropriate surrogates for primary cells isolated from individuals and accurately reflect the response and the pathways involved in normal cells. Likewise, it is unclear whether genetic lessons gleaned from single experiments in PBMC from a small number of individuals faithfully reflect innate differences in damage response pathways, or whether differences in IR response merely reflect stochastic effects having little to do with biological inter-individual differences.

To distinguish between these possibilities and to establish a platform by which results obtained in LCL can be interpreted in the context of normal cells, we performed the largest study to date of the apoptotic response to IR in PBMC freshly isolated from healthy human volunteers. We observed tremendous variability in the apoptotic response to IR among individuals, and found that this response was highly reproducible within an individual, suggesting it is not stochastic. In addition, we found that IR-induced apoptosis differed significantly between males and females, with the response in males inversely associated with age.

Materials and Methods

Subjects

Healthy individuals participating in this study were volunteers recruited from staff at two separate medical centers. Subjects were excluded only for the following reasons: 1) a personal history of cancer or autoimmune disease, 2) systemic steroid use within the past year, or 3) a viral or bacterial illness within the past month. Individuals not excluded were sequentially assigned a study ID; a total of 90 subjects were recruited. Subjects 1–50 were accrued and investigated at one site and are referred to as Cohort 1. Subjects 51–90 were accrued and investigated at the other site and are referred to as Cohort 2. This study was approved by the local institutional review boards and informed consent was obtained from all study participants.

PBMC isolation and irradiation

Subjects were phlebotomized between 9–11 am and whole blood (30 ml) collected in heparinized tubes. PBMC were isolated by ficoll gradient centrifugation (Ficoll-Paque Premium, GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA), washed in PBS, and resuspended in RPMI 1640 medium containing 10% serum at a density of $1 \times 10^6/\text{ml}$. Cells were then divided into two batches, one subjected to a 5 Gy dose at room temperature using a Cesium-137 source irradiator and the other sham-treated (0 Gy). Both irradiated and sham-treated samples were incubated for 24 hours at 37°C, 5% CO₂. For all subjects, the time range from blood draw to irradiation or sham-treatment was 2–3 hours.

Samples from Cohort 1 were irradiated using a Mark I, Model 68 Irradiator (JLS Shepherd and Assoc., San Fernando, CA, USA) at a dose rate of 10 Gy/min. Samples from Cohort 2 were irradiated using a GammaCell 1000 irradiator (Best Theratronics, Ottawa, Ontario, CA) at a dose rate of 8 Gy/min.

Measurement of apoptosis

For each subject, apoptosis was measured by TUNEL in three samples: 1) at 0 hours immediately prior to irradiation; 2) at 24 hours following irradiation; and 3) at 24 hours following sham-treatment. The 24 hour time point was chosen based on preliminary experiments to determine the time point of maximal difference in apoptosis between irradiated and sham-treated samples.

For TUNEL, 1×10^6 cells were washed in PBS, incubated for 1 hour at room temperature in 2% formaldehyde diluted in PBS to cross-link DNA, then washed again and fixed in ice cold 80% ethanol for at least 24 hours. After two PBS washes to remove ethanol, DNA strand breaks were measured by on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) using the *In situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Indianapolis, IN, USA), counting at least 10,000 cells. All analysis was performed using CellQuest software (BD Biosciences, San Jose, CA, USA). For each subject, the 0 hour samples were fixed and then subsequently processed and analyzed in tandem with the two 24 hour samples.

The 0 hour sample was used exclusively for quality control to ensure that samples were not damaged by phlebotomy and immediate post-phlebotomy processing prior to irradiation or sham-treatment. Final IR-induced apoptosis is reported as the percent apoptosis at 24 hours following sham-treatment subtracted from the percent apoptosis at 24 hours following irradiation.

Replication analysis

To investigate the reproducibility of IR-induced apoptosis, ten individuals in Cohort 1 spanning the entire range of IR-induced apoptosis were tested an additional two times ($n = 3$ total), with 3–6 months separating each biological replicate, and using the same Mark I, Model 68 irradiator. For each of these 10 individuals, only the results of the first blood draw were used for analysis with the entire cohort.

Statistical Analysis

For all analyses, the dependent variable was the difference between apoptosis measured 24 hours after irradiation and 24 hours after sham-treatment; we refer to this simply as apoptosis at 24 hours after irradiation, or “IR-induced apoptosis.”

In our models, we considered age, race, and sex as factors potentially modifying IR-induced apoptosis. We also considered cohort as a factor because of possible confounding effects due to, for example, differences in dosimetry, dose rate, or dose delivery between cohorts. To investigate the contribution of these variables to inter-individual variability in apoptosis, we performed forward-backward stepwise regression using Akaike Information Criteria (AIC)(Akaike 1974). Variables that maximally decreased AIC were added to the model as covariates, and previously added variables were excluded if doing so decreased AIC. Model building stopped when no further decrease in AIC was possible. Regression parameters and means were calculated relative to an average subject with respect to age, sex, and cohort by using centered covariate values (subtracting the mean covariate value from each individual covariate value.)

To assess the influence of cohort on apoptosis measured at 0 hours and at 24 hours after sham-treatment, we used a Wilcoxon Rank Sum test (Wilcoxon 1946). We tested for heteroscedasticity of apoptosis as a result of cohort using an F-test based on the error variances estimated from the cohort-specific regressions.

All analyses were performed in *R* (R Core Team, *R: A Language and Environment for Statistical Computing*, Vienna, Austria, 2013, <http://www.R-project.org/>).

Results

We analyzed IR-induced apoptosis in fresh PBMC isolated from 90 healthy individuals from two independent cohorts, Cohort 1 ($n = 50$) and Cohort 2 ($n = 40$). 47 individuals were male and 43 female; 65 were self-reported as of European ancestry, 8 as African American, and 17 as Asian; age ranged from 22–60 years (Table IA).

To quantify IR-induced apoptosis, we subtracted spontaneous apoptosis at 24 hours from apoptosis at 24 hours following a dose of 5 Gy. We observed an unexpected degree of variability in IR-induced apoptosis among individuals, ranging from 21–62%; (mean = 36.1%, $se = 1.1$). This heterogeneity was reproducibly observed in both Cohort 1 and Cohort 2; neither mean IR-induced apoptosis nor standard deviation differed between cohorts (mean_{Cohort1} = 37.4% vs. mean_{Cohort2} = 34.5%, $p_{\text{means}} = 0.19$; sd_{Cohort 1} = 10.9, vs. sd_{Cohort 2} = 9.2; $p_{\text{sdevs}} = 0.59$) (Figure 1; Table IB).

For all individuals except two, apoptosis at 0 hours was 0–1%. For all individuals except four, spontaneous apoptosis at 24 hours after sham-treatment was <10%, suggesting that PBMC undergo low levels of apoptosis when removed from their normal environmental milieu. Although apoptosis in Cohort 2 at 0 hours and at 24 hours following sham-treatment was significantly different than in Cohort 1, the actual difference between the means of both cohorts was small (for 0 hours: mean_{cohort1} = 0.24%, mean_{cohort2} = 0.93%, mean_{Cohort1} –

mean_{Cohort2} = -0.69%, $p_{\text{Wilcoxon}} = 2.3 \times 10^{-13}$; for 24 hours sham: mean_{cohort1} = 6.34%, mean_{cohort2} = 5.33%, mean_{Cohort1} - mean_{Cohort2} = 1.01%, $p_{\text{Wilcoxon}} = 0.04$). Importantly, we did not observe a correlation between apoptosis at 0 hours and sham-treatment at 24 hours ($r = -0.20$, $p = 0.06$), IR-induced apoptosis at 24 hours ($r = -0.08$, $p = 0.43$), or IR-induced apoptosis ($r = -0.04$, $p = 0.72$). Similarly, apoptosis 24 hours after sham-treatment was not correlated with IR-induced apoptosis ($r = 0.11$, $p = 0.28$). Taken together, these results suggest that samples with higher levels of spontaneous apoptosis were not “primed” to have higher levels of IR-induced apoptosis and that cohort effects did not contribute significantly to our results. A fuller investigation of the impact of cohort on apoptosis is provided in the Supplementary Materials.

The observed inter-individual variability in IR-induced apoptosis could reflect either intrinsic differences among individuals or stochastic effects. To distinguish between these possibilities, ten volunteers were selected for repeated sampling ($n = 3$). In each case, individuals initially found to have low levels of IR-induced apoptosis always had low levels of IR-induced apoptosis, and individuals found to have high levels of IR-induced apoptosis always had high levels of IR-induced apoptosis (Figure 2).

We then investigated the influence of different factors on IR-induced apoptosis using forward-backward selection. Neither race ($p = 0.75$) nor age ($p = 0.13$) was, as a single variable, associated with apoptosis (Table II). Surprisingly, however, we found that males and females differed significantly in their susceptibility to IR-induced apoptosis (Mean: 41.0% vs. 30.7%; $p = 3.5 \times 10^{-7}$; Table II). Females were clustered at low levels of apoptosis, whereas males were widely dispersed; indeed, all 14 individuals with apoptosis levels > 45% were males (Figure 3, Supplementary Table I).

After conditioning on sex, we found that including age and cohort improved model fit (Table III, Supplementary Table II). When fitting sex-specific linear age effects, we found that younger males had higher levels of IR-induced apoptosis than older males ($b_{\text{Age, Male}} = -0.33\%$, $p = 0.01$), but that no such age effect was observed in females ($b_{\text{Age, Female}} = -0.03\%$, $p = 0.82$) (Table III, Figure 4). We also found that males younger than 40 had markedly higher levels of apoptosis compared to age-matched females (mean: 41.7% vs. 28.8%; $p = 4.2 \times 10^{-7}$). The level of apoptosis in males and females older than 40 was much more similar although males still had higher levels (mean: 35.0% vs. 29.0%; $p = 0.06$). These results are summarized in Table III. To ensure these results were not confounded by ancestry differences between males and females, the analysis was repeated in individuals of European descent alone, with similar results (Supplementary Table III).

Discussion

The major findings of this study are twofold. First, we found that IR-induced apoptosis varies dramatically in PBMC isolated from healthy volunteers, but is highly reproducible within a single individual. This reproducibility supports the use of PBMC as a high-fidelity model of cellular IR-induced processes without the inherent questions raised by cell lines such as immortalized LCL. Second, we found that males and females differ significantly in their apoptotic response to IR, with age modifying this response in males but not females.

Younger males had the highest levels of IR-induced apoptosis; as males aged, average levels of IR-induced apoptosis decreased, ultimately becoming similar to, but still slightly greater than, those observed in females. Taken together, our results suggest the existence of sex-specific modifiers of inborn apoptotic pathways.

We speculate that one modifying factor may be inter-individual differences in sex hormone levels. That sex hormone levels influence apoptosis is well-established (Grimaldi et al. 2002, Jin et al. 2006, Morkuniene et al. 2006, Vasconsuelo et al. 2011). The influence of the interaction between sex hormones and innate apoptotic programs on cancer risk is demonstrated by the example of the *MDM2* SNP309, which is associated with a wide variety of cancers, but only in premenopausal women (Gold et al. 2004, Bond et al. 2006, Ellis et al. 2008). *MDM2* is a critical negative regulator of the p53 tumor suppressor. In individuals with the risk allele, an enhancer site is created by which estrogen receptor can induce *MDM2*. Higher levels of *MDM2* result in lower levels of p53, and concomitantly, less efficient tumor suppression (Bond et al. 2004).

One possible limitation of our study is that we did not obtain cell counts and differentials for the subjects analyzed. Average white blood cell (WBC) counts differ among races and the proportion of WBC subtypes varies with age and sex (Tollerud et al. 1989, Utsuyama et al. 1992). It is possible that different cell types are differentially susceptible to radiation; if so, then it is possible that the observed inter-individual heterogeneity in IR-induced apoptosis among healthy individuals and between males and females may actually reflect differences in the mix of WBC types rather than differences in the efficiency of radiation response pathways. We argue, however, that because WBC counts and differentials are highly variable not only among individuals but also within a single individual (Lacher et al. 2012), if differences in WBC populations contributed significantly to our results, then we should have observed variability in the apoptotic response of our biological replicates to radiation. That this was not the case suggests that differences in the mix of cell types among individuals or differences in susceptibility to radiation-induced apoptosis among WBC subtypes do not explain our results.

In conclusion, our findings indicate that IR-induced apoptosis in freshly isolated human PBMC may be a useful and highly reproducible phenotype to study inter-individual differences in apoptosis. In contrast to studies performed in LCL, we were able to discern sex and age-specific effects, which may be of importance for understanding the etiology of sex and age-specific cancer and autoimmune disease associations. Future studies integrating genetic and functional investigations in both PBMC and LCL will be required to fully assess the implications of these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. IR-induced apoptosis in PBMC isolated from healthy individuals is highly variable
Plots of IR-induced apoptosis showing the median, first, and third quartiles and largest and smallest data points within 1.5 times the interquartile range for Cohort 1, Cohort 2 and the combined cohort.

Figure 2. IR-induced apoptosis is highly reproducible within a single individual

Apoptosis induced by 5 Gy IR was measured in three independent experiments in ten individuals spanning the entire range of IR-induced apoptosis.

Figure 3. IR-induced apoptosis in healthy volunteers differs significantly between males and females

IR-induced apoptosis is plotted by 5-percentile bins separately for males (dark) and females (light) stratified by Cohort 1 (Figure 3A) and Cohort 2 (Figure 3B), with similar results.

Figure 4. IR-induced apoptosis declines significantly with age in males but not in females
IR-induced apoptosis values are plotted along with a best-fit line for males (black) and females (grey) found by regressing apoptosis on sex, cohort, and sex-specific age. Results demonstrate a significant association with age in males ($p = 0.01$) but not in females ($p = 0.82$).

Table I

Demographics (Table IA) and the distribution of IR-induced apoptosis (Table IB) stratified by cohort.

	1A Demographics		
	Cohort 1 (n=50)	Cohort 2 (n=40)	p-value
Male (n (%))	22 (44.0)	25 (62.5)	0.13
Age (years)			
Mean	35.6	35.0	0.79
Range	22 – 60	22 – 60	
Ancestry (n (%))			
European	39 (78.0)	26 (65.0)	0.26
Non-European	11 (22.0)	14 (35.0)	

	1B Distribution of IR-Induced Apoptosis		
	Cohort 1 (n=50)	Cohort 2 (n=40)	p-value
IR-Induced Apoptosis (%)			
20–25	7	9	0.81
26–30	7	5	
31–35	12	9	
36–40	8	7	
41–45	7	5	
46–50	0	1	
51–55	5	4	
56–60	2	0	
61+	2	0	
Mean	37.4	34.5	0.19
Std Dev	10.9	9.2	0.59
Range	21 – 62	21 – 55	

Table II

Relationship between IR-induced apoptosis and sex, ancestry, and age.

Characteristic (n)	Mean Apoptosis (se) *	p-value
Sex		
Male (47)	41.0% (1.3)	3.5×10^{-7}
Female (43)	30.7% (1.3)	
Ancestry		
European (65)	35.8% (1.3)	0.75
Asian (17)	35.9% (2.8)	
African American (8)	38.7% (3.8)	
Age		
22–30 (28)	40.3% (2.1)	0.13
31–40 (41)	37.4% (1.8)	
41–50 (12)	32.9% (3.0)	
51–60 (9)	34.5% (3.5)	

* All means and standard errors were calculated using only an intercept and the variable listed. That is, the means and standard errors are not adjusted for cohort, sex, or age.

Table III

Multivariate analysis of factors influencing IR-induced apoptosis.

Parameter estimates for age, sex, and cohort are from a model that regresses IR-induced apoptosis on those variables. Parameter estimates for ancestry is from a model that fits age, sex, cohort, and ancestry (as a categorical variable). The parameter estimates for African Americans and Asians are the difference in the mean apoptosis levels between these groups and European Americans. The p-values are from two-sided Wald tests except in the case of ancestry in which case it is from a likelihood ratio test.

Variable	Change in apoptosis per unit change in variable *	Standard Error	p-value **
Sex (Male)	11.07	1.82	3.4×10^{-8}
Cohort	-4.88	1.83	0.01
Race			0.45
African American	4.02	3.18	0.21
Asian	0.28	2.40	0.91
Age	-0.18	0.09	0.05
Age in Males	-0.33	0.13	0.01
Age in Females	-0.03	0.13	0.82
Sex (Male) when Age < 40	12.87	2.27	4.2×10^{-7}
Sex (Male) when Age > 40	6.00	2.98	0.06

* All models are fit including sex, cohort, and age

** p-values are from the Wald test for the regression parameter when IR-induced apoptosis is regressed on sex, cohort, and age.