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Identifying a Diagnostic Window for the Use of Gene Expression Profiling to Predict Acute Radiation Syndrome

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In the event of a mass casualty radiological or nuclear scenario, it is important to distinguish between the unexposed (worried well), low-dose exposed individuals and those developing the hematological acute radiation syndrome (HARS) within the first three days postirradiation. In previous baboon studies, we identified altered gene expression changes after irradiation, which were predictive for the later developing HARS severity. Similar changes in the expression of four of these genes were observed using an *in vitro* human whole blood model. However, these studies have provided only limited information on the time frame of the changes after exposure in relationship to the development of HARS. In this study we analyzed the time-dependent changes in mRNA expression after *in vitro* irradiation of whole blood. Changes in the expression of informative mRNAs (*FDXR*, *DBB2*, *POU2AF1* and *WNT3*) were determined in the blood of eight healthy donors (6 males, 2 females) after irradiation at 0 (control), 0.5, 2 and 4 Gy using qRT-PCR. *FDXR* expression was significantly upregulated ($P < 0.001$) 4 h after $\geq$0.5 Gy irradiation, with an 18–40-fold peak attained 4–12 h postirradiation which remained elevated (4–9-fold) at 72 h. *DDB2* expression was upregulated after 4 h (fold change, 5–8, $P < 0.001$ at ≥ 0.5 Gy) and remained upregulated (3–4-fold) until 72 h ($P < 0.001$). The earliest time points showing a significant downregulation of *POU2AF1* and *WNT3* were 4 h (fold change = 0.4, $P = 0.001$, at 4 Gy) and 8 h (fold change = 0.3–0.5, $P < 0.001$, 2–4 Gy), respectively. These results indicate that the diagnostic window for detecting HARS-predictive changes in gene expression may be opened as early as 2 h for most (75%) and at 4 h postirradiation for all individuals examined. Depending on the RNA species studied this may continue for at least three days postirradiation.

INTRODUCTION

In the event of a mass casualty radiological emergency the evaluation of exposed individuals is necessary to both predict the severity of radiation injuries and to prioritize appropriate treatment (*1*). A high-throughput evaluation is required for incidents involving hundreds or thousands of individuals, for instance widespread contamination after the detonation of a dirty bomb, as limited clinical resources make it important to identify the few highly exposed individuals from the many who believe they were exposed (the worried well).

The hematological acute radiation syndrome (HARS) is a life-threatening consequence of high-dose exposure. The clinical course of HARS results in a decrease in granulocytes and thrombocytes in the peripheral blood, leading to immune suppression and hemorrhage in the weeks postirradiation. To implement pre-emptive treatment, e.g., support of the hematologic system (*2, 3*), testing must ideally be performed as soon as possible after exposure, preferably within 1–3 days, even before symptoms are evident.

METREPOL [MEdical TREatment ProtocOLs (*4*)] categorizes HARS into five severity classifications based on blood cell count changes in the weeks after exposure: no HARS (H0), low (H1), medium (H2), severe (H3) and fatal (H4) HARS. Only H2–4 HARS require immediate hospitalization and intensive therapy. Long-term surveillance after an H1 HARS diagnosis classification is recommended, because of an increased risk for chronic diseases such as cancer or non-cancer health outcomes (e.g., cardiovascular disease) (*5*).

We recently identified a candidate set of gene transcripts expressed in the peripheral blood of baboons within 1–2 days postirradiation. The candidate markers were subse-

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quently validated in irradiated human patients examined 24 h postirradiation (*6, 7*). Analysis of the changes in expression of this gene set allowed us to distinguish between irradiated individuals who had developed H1 and those developing the more severe H2–4 HARS (*6, 8, 9*). In particular, changes in expression of *WNT3* and *POU2AF1* were predictive for H2–4 severity, while *FDXR* and *DDB2* expression changes were limited to lower dose exposures that did not result in severe HARS (*10*). Thus, upregulated *FDXR* or *DDB2*, combined with downregulated *WNT3* and *POU2AF1*, reflects a more severe (H2–4) HARS, while upregulated *FDXR* or *DDB2* and unaltered *WNT3* and *POU2AF1* would be indicative for a radiation-induced mild (H1) HARS severity. Noteworthy, in this already-published work, we focused on the gene-to-disease (effect) relationship rather than a dose-to-gene expression relationship (dose estimation). Our goal has been to predict the effect (disease) based on early changes in gene expression after irradiation.

These studies do not indicate the predictive potential of the markers over an extended (three-day) diagnostic window. In particular, systematic examinations with many time points are missing, so as to determine how early the changes occur after exposure, or if observed changes persist during the diagnostic window so that they can be used even on day 3 after exposure. Since such questions cannot be answered through *in vivo* studies, we established a model system for the *in vitro* exposure of whole blood. Significantly, in this model the altered expression of *FDXR*, *DDB2*, *WNT3*, *POU2AF1* replicates our observations in irradiated baboons and humans (*11*). We now report that the changes in marker gene expression in this model indicate that they retain a predictive function over a three-day postirradiation window.

MATERIALS AND METHODS

Sample Collection, Irradiation and Cell Culture

Peripheral whole blood from eight healthy donors (six males, two females, aged 23–58) was collected into Vacutainer® EDTA blood tubes, then either sham or 0.5 Gy irradiated (related to H1 HARS severity), 2 or 4 Gy (related to H2–4 HARS severity). Single-dose X-ray irradiation was performed *in vitro* at 37°C. X rays were delivered using 3-mm beryllium and 3-mm aluminum filters to give a mean photon energy of 100 keV delivered at approximately 1.0 Gy/min (Maxishot SPE cabin; Yxlon, Hamburg, Germany). The absorbed doses were measured using a UNIDOS webline 10021 dosimeter (PTW, Freiburg, Germany). The dose rate was approximately 1.0 Gy/min at 13 mA and accelerating potential 240 kV (maximum photon energy of 240 keV). Previously reported data for RNA quantity, quality, as well as gene expression in both the baboon and human studies influenced the choice of dose. Irradiated blood samples were diluted 1:1 in prewarmed RPMI cell culture media supplemented with 10% fetal calf serum and aliquoted for analysis of gene expression after incubation for 0, 1, 2, 4, 8, 16, 24, 48 and 72 h at 37°C and a pH of 7.4. Nucleated cell counts were performed at each time point using a Neubauer counting chamber.

RNA Extraction and Quality Control

RNA was isolated using the QIAamp® RNA Blood Mini Kit (QIAGEN®, Valencia, CA) following the manufacturer's protocol, but with a doubling of the centrifugation time to increase yield. In brief, one volume of whole blood was mixed with five volumes of the erythrocyte lysis (EL) buffer, incubated on ice and centrifuged for 20 min at 400*g* and 4°C. After discarding the supernatant, this step was repeated with another two volumes of EL buffer. White blood cells of the pellet were lysed (RLT buffer), homogenized, ethanol was added, the samples were applied to the QIAamp membrane spin column, wash buffer was added and samples spun. DNA was digested (DNase) on the column, samples washed several times and RNA eluted in RNAse-free water. The isolated RNA was quantified spectrophotometrically (NanoDrop™; PeqLab Biotechnology, Erlangen, Germany). RNA integrity was assessed using the 2100 Agilent Bioanalyzer (Life Science Group, Penzberg, Germany). Possible contamination by sample genomic DNA was controlled by PCR using primers for the actin gene. RNA specimens with a ratio of A260/A280 nm $\geq$2.0 and RNA integrity number (RIN) $\geq$8 were processed for qRT-PCR analysis.

qRT-PCR

Aliquots of total RNA (e.g., 0.25, 0.5 or 1 μg) were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Life Technologies, Darmstadt, Germany). The PCR reactions were performed using commercial TaqMan® assays (*FDXR*, Hs01031617_m1; *WNT3*, Hs00902257_m1; *POU2AF1*, Hs01573369_m1; *DDB2*, Hs03044949_m1; 18S rRNA (Human), Hs03003631_g1) following the qRT-PCR 7900er amplification protocol. The cycle threshold (Ct) values of the four genes were normalized relative to 18S rRNA. The ratio/fold change relative to the nonirradiated sample at the same time point was determined. A fold change of one corresponds to a gene expression similar to nonirradiated samples. A fold change higher or lower than one refers to a several-fold over- or under-expression of the gene of interest after irradiation relative to the reference. All materials and consumables used were acquired from Thermo Fisher/Applied Biosystems (Weiterstadt, Germany). All experimental work was performed following standard operating procedures at the DIN-accredited laboratories of the Bundeswehr Institute of Radiobiology (DIN EN ISO 9001/2008).

Statistical Analysis

Statistics where performed using the Statistical Analysis System (SAS®), version 9.4, (Cary, NC) or SigmaPlot™ 14 (Jandel Scientific, Erkrath, Germany). Group comparisons were performed using either parametrical (Student's *t* test, Welch's *t* test) or non-parametrical tests (Kruskal-Wallis) or linear and linear-quadratic regression analysis, where applicable (e.g., examining for normal distribution and equal variance). Data are reported as mean ± standard error of mean.

RESULTS

Cell Culture Model

White blood cell counts (WBCC) in unirradiated whole blood remained stable for the first 12 h ($P = 0.97$, Kruskal-Wallis), but subsequently decreased in a linear fashion ($3.5 \times 10^6 \pm 0.5$/ml at 1 h to $0.7 \times 10^6 \pm 0.1$/ml at 72 h of cell culture, $P < 0.0001$, Fig. 1). A similar pattern was also observed in the blood cultures irradiated at 0.5, 2 and 4 Gy and the number of WBCC were not altered by radiation (data not shown).

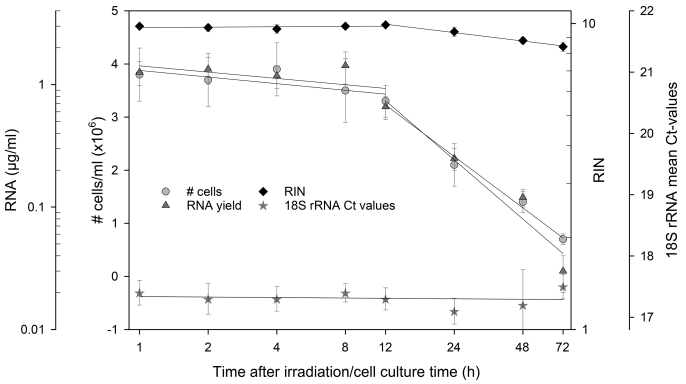

FIG. 1. Examinations on changes in whole blood cell counts (inner y-scale to the left), isolated RNA yields (outer y-scale to the left), corresponding RNA quality (RIN, inner y-scale to the right) and housekeeping gene expression changes (18S rRNA, outer y-scale to the right) over cell culture time. RIN = RNA integrity number; 18S rRNA = 18S ribosomal RNA; Ct = cycle threshold.

RNA yields decreased in a linear manner over time in culture, starting after 8 h ($P < 0.0001$). In agreement with the decline in cell numbers, decreased mean RNA yields were observed [from 1.4 ± 0.3 µg/ml at 1 h to 0.03 ± 0.01 µg/ml at 72 h of cell culture (Fig. 1)]. These quantitative changes appeared after irradiation as well, but did not aggravate due to exposure (data not shown).

A slight decrease of RIN values, from 9.3 ± 0.4 at 1 h to 8.4 ± 0.3 at 72 h of cell culture, was observed after sham exposure and irradiation and started after 12 h (Fig. 1). RIN values before 12 h appeared constant ($P = 0.63$, Kruskal-Wallis) and declined linearly afterwards ($P = 0.001$).

Mean 18S rRNA Ct values of 17.3 did not change over cell culture time and after sham irradiation or irradiation (Fig. 1).

Differential Gene Expression and Association with Dose over Cell Culture Time

Previous *in vivo* examinations in 17 baboons and five leukemia patients examined at 24 h and/or 48 h postirradiation are added to the panels/genes of Fig. 2 (white filled diamonds in the shadowed gray area) to reflect the similarity in gene expression changes measured at this period of time *in vivo* and in comparison to our actual *in vitro* measurements. Based on this similarity, we examined how the diagnostic window can be expanded before 24 h and after 48 h of radiation exposure.

A statistically significant upregulation of *FDXR* expression was observed 4 h postirradiation at all three tested

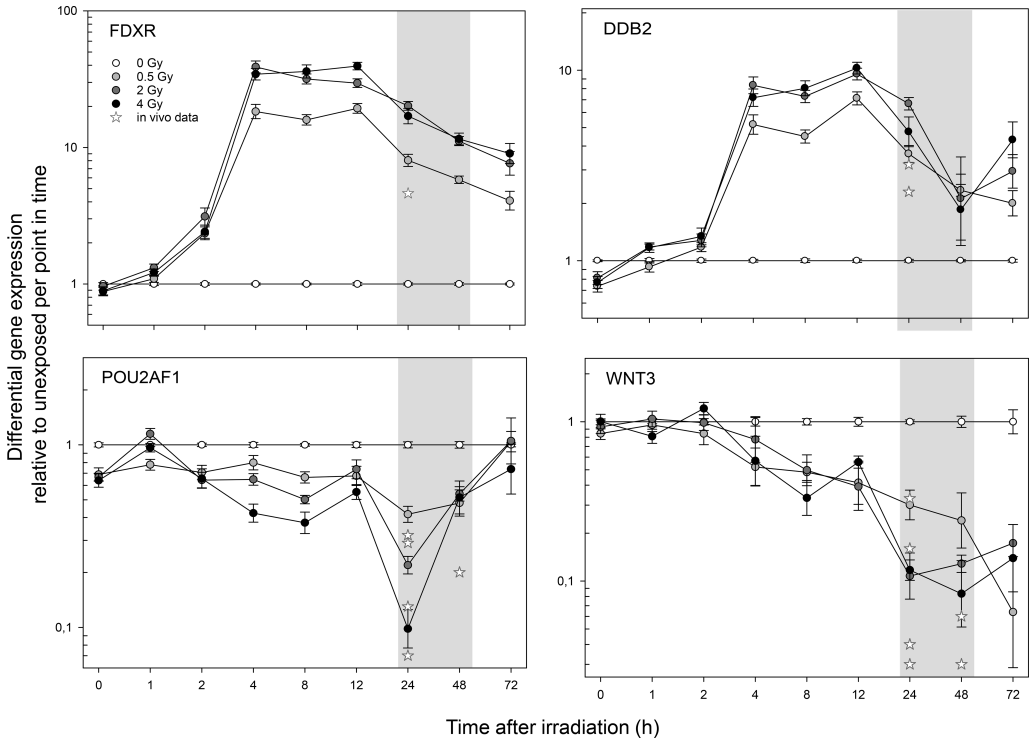

FIG. 2. Differential gene expression of *FDXR*, *DDB2*, *POU2AF1* and *WNT3* is shown in separate panels over the time after irradiation (cell culture). Fold changes are calculated with the nonirradiated samples of the same time point used as a reference (set to the value of 1) to compensate for methodological variance. Symbols reflect the geometrical mean and error bars the standard error of mean (n = 8). Individual plots per donor and gene are shown in Supplementary Figs. 1–4 (https://doi.org/10.1667/RADE-20-00126.1.S1). The superimposed gray areas refer to the 24–48 h time frame and include previously published *in vivo* measurements (fold changes relative to nonirradiated, white stars), originating either from baboons or leukemia patients (values are taken from Table 2).

TABLE 1
Summary on the Suggested Onset, Maximum and End of the Diagnostic Window for Hematological Acute Radiation Syndrome (HARS) Prediction based on Fold Changes in Gene Expression Examined in Four Genes over Time after Irradiation

Gene	Onset (h)	Fold changes (*P* value)	Individuals (n/%)	Maximum (h)	Fold changes (*P* value)	End (h)	Fold changes (*P* value)	Dose (Gy)
FDXR	4	18.3 (<0.001)	8/100	4–12	18.2–19.2 (<0.001)	72	4.1 (<0.001)	0.5
	4	38.8 (<0.001)	8/100	4–12	30.0–39.7 (<0.001)	72	7.6–9.2 (<0.001)	2.0, 4.0
DDB2	4	5,2 (<0.001)	8/100	4–12	5.3–7.0 (<0.001)	72	2.0 (<0.001)	0.5
	4	7.2–8.4 (<0.001)	8/100	4–12	7.2–10.2 (<0.001)	72	3.0–4.3 (<0.001)	2.0, 4.0
POU2AF1	8	0,5 (<0.001)	7/87.5	24	0.2 (<0.001)	48	0.5 (<0.001)	2.0
	4	0,4 (0.001)	7/87.5	24	0.1 (<0.001)	48	0.5 (0.007)	4.0
WNT3	8	0.3–0.5 (<0.001)	7/87.5	8–48	0.3–0.1 (≤0.001)	72	0.2–0.1 (na, 0.04)	2.0, 4.0

Notes. Doses corresponding to the gene expression changes are shown in the right-side column. These values are taken from Fig. 2. Corresponding to the fold changes examined at a certain time (onset) and dose, we added the number of individuals and percentage (after the dash) where these radiation-induced changes were observed.

doses (Fig. 2 and Table 1). The levels continued to increase, reaching a peak 4–12 h postirradiation with increases of 18–40-fold over controls. Thereafter, levels gradually decreased, but even after 72 h there remained an average of an eightfold upregulation (Fig. 2 and Table 1). Similar patterns were observed in all eight healthy donors (Supplementary Fig. S1; https://doi.org/10.1667/RADE-20-00126.1.S1) and the inter-individual variance was low (Fig. 1). These fold changes increased dose dependently at 2 h and at later time points following a linear-quadratic function and showed a saturation of fold changes at ≥2 Gy (Fig. 3).

The expression profile of *DDB2* after irradiation showed characteristics similar to those of *FDXR*, with an increase over time and dose followed by a gradual decline while still remaining elevated after 72 h (Figs. 2 and 3). However, unlike *FDXR*, the increase in *DDB2* was not yet significant at 2 h postirradiation, but rose to a 5–10-fold increase at 4–12 h postirradiation. Moreover, unlike *FDXR* the later time points were somewhat variable, with

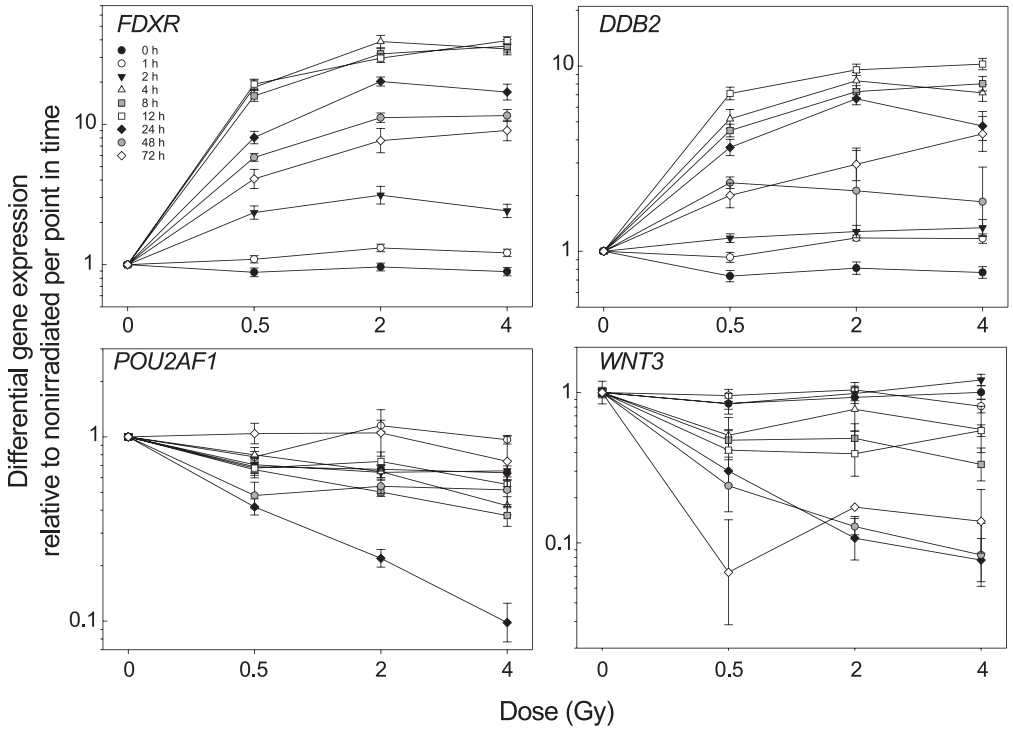

FIG. 3. A dose-response relationship for differential gene expression of *FDXR*, *DDB2*, *POU2AF1* and *WNT3* is shown in separate panels and for each examined time point after irradiation (cell culture). Fold changes are calculated with the nonirradiated samples of the same time point used as a reference (set to the value of 1) to compensate for methodological variance. Symbols reflect the geometrical mean and error bars the standard error of mean (n = 8).

TABLE 2
Overview of Cited Work Regarding Onset and Fold Changes in Gene Expression after Radiation Exposure Examined either *In Vivo* or *In Vitro* Experiments

				Materials and methods						
Onset (h)	Dose/ Fold changes	Duration (h)	Dose/ Fold changes (max)	No. of donors	Conditions	Materials	Dose (Gy)	Time points (h)	Platform	Ref.
FDXR										
2	3[a]	2–24	6[a]	2 Females	*Ex vivo, in vitro*	T-lymphocyte cultures	2 Gy	0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 24	qRT-PCR	(*19*)
2	0.5 Gy/3[a]	2–24	0.5 Gy/15[a]	17 Females,	*Ex vivo, in vitro*	EDTA whole blood	0.5, 1, 2, 4,	2, 24	qRT-PCR	(*10*)
				9 males						
	1 Gy/3[a]		1 Gy/28[a]							
	2 Gy/4[a]		2 Gy/37[a]							
	4 Gy/6[a]		4 Gy/46[a]							
4	14.6	-	-	8 Patients	*In vivo*	Whole blood	1.25	4	Microarray	(*13*)
4	1.25 Gy/13	4–24	1.25 Gy/13	18 Patients	*In vivo*	Whole blood	1.25, 3.75 (3 fractions)	4, 24	Microarray, qRT-PCR	(*14*)
	1.25 Gy/16[a]		1.25 Gy/16[a]							
6	0.5 Gy/7[a]	6–24	0.5 Gy/7[a]	5 Males,	*Ex vivo, in vitro*	Whole blood	0.5, 2, 5, 8	6, 24	qRT-PCR	(*12*)
				5 females		(1:1 RPMI 1640/ 10% FBS)				
	2 Gy/12[a]		2 Gy/12[a]							
	5 Gy/15[a]		5 Gy/15[a]							
	8 Gy/21[a]		8 Gy/21[a]							
6	0.5 Gy/2.5[a]	6–48	0.5 Gy/22[a]	3 Males,	*Ex vivo, in vitro*	Lymphocyte cultures	0.5, 1, 2, 4	6, 24, 48	qRT-PCR	(*16*)
				3 females						
	1 Gy/3[a]		1 Gy/25[a]							
	2 Gy/3.5[a]		2 Gy/30[a]							
	4 Gy/4[a]		4 Gy/35[a]							
8	0.5 Gy/14[a]	8–48	0.5 Gy/14[a]	3 Males,	*Ex vivo, in vitro*	EDTA whole blood	0.5, 1, 2	8, 12, 24, 36, 48	qRT-PCR	(*18*)
				4 females						
	1 Gy/21[a]		1 Gy/21[a]							
	2 Gy/22		2 Gy/22							
8	1 Gy/25	8–24	1 Gy/25	5 Males,	*Ex vivo, in vitro*	PBMC cultures	1	8, 24	qRT-PCR	(*17*)
				5 females		(LGM-3)				
	1 Gy/19[a]		1 Gy/19[a]			Whole blood				
8	0,15 Gy/1.0	8–24	0,15 Gy/1.0	4 Males,	*Ex vivo, in vitro*	Expanded T cells	0.15, 12	3, 8, 24	Microarray	(*15*)
				4 females						
	12 Gy/3,82		12 Gy/3,82			(X-vivo 15/5% FBS)				
24	2–5 Gy/5.8	-	-	15 Patients	*In vivo*	Whole blood	2–5	24	qRT-PCR	(*7*)
24	1 Gy/4	-	-	5 Patients	*Ex vivo, in vitro*	Whole blood	1	24	qRT-PCR	(*7*)
	2.5 Gy/12.3						2.5			
	4 Gy/12.9						4			
DDB2										
3	2,5[a]	2–24	3[a]	2 Females	*Ex vivo, in vitro*	T-lymphocyte cultures	2 Gy	0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 24	qRT-PCR	(*19*)
4	5.95	-	-	8 Patients	*In vivo*	Whole blood	1.25	4	Microarray	(*13*)
4	1.25 Gy/5.5	4–24	1.25 Gy/5.5	18 Patients	*In vivo*	Whole blood	1.25, 3.75 (3 fractions)	4, 24	Microarray, qRT-PCR	(*14*)
	1.25 Gy/5.5[a]		1.25 Gy/5.5[a]							
8	0.5 Gy/5[a]	8–48[a]	0.5 Gy/5[a]	3 Males,	*Ex vivo, in vitro*	EDTA whole blood	0.5, 1, 2	8, 12, 24, 36, 48	qRT-PCR	(*18*)
				4 females						
	1 Gy/5.8[a]		1 Gy/5.8[a]							
	2 Gy/6[a]		2 Gy/6[a]							
8	1 Gy/5[a]	8–24	1 Gy/5[a]	5 Males,	*Ex vivo, in vitro*	PBMC cultures	0.1, 1	8, 24	qRT-PCR	(*17*)
				5 females		(LGM-3)				
	1 Gy/5.5[a]		1 Gy/5.5[a]			Whole blood				
8	0,15 Gy/1	8–24	0,15 Gy/1	4 Males,	*Ex vivo, in vitro*	Expanded T cells	0.15, 12	3, 8, 24	Microarray	(*15*)
				4 females		(X-vivo 15/5% FBS)				
	12 Gy/1.98		12 Gy/1.64							

Continued on next page

TABLE 2
Continued.

Onset (h)	Dose/ Fold changes	Duration (h)	Dose/ Fold changes (max)	Materials and methods						Ref.
				No. of donors	Conditions	Materials	Dose (Gy)	Time points (h)	Platform	
24	0.5 Gy/6[a]	-	-	17 Females, 9 males	*Ex vivo, in vitro*	EDTA whole blood	0.5, 1, 2, 4,	2, 24	qRT-PCR	(*10*)
	1 Gy/7[a]									
	2 Gy/10[a]									
	4 Gy/9[a]									
24	2–2.5 Gy/2.2	-	-	18 Baboons,	*In vivo*	Whole blood	2–2.5	24	qRT-PCR	(*7*)
	4–5 Gy/2.3			18 Baboons,			4–5			
	2–5 Gy/3.2			15 Patients			2–5			
24	2.5 Gy/2.6	-	-	5 Baboons	*Ex vivo, in vitro*	Whole blood	2.5	24	qRT-PCR	(*7*)
	4 Gy/3.3			5 Baboons			4			
	1 Gy/2.44			5 Patients			1			
	2.5 Gy/3			5 Patients			2.5			
	4 Gy/2.8			5 Patients			4			
WNT3										
24	2.5–5 Gy/0.04	24–48	-	18 Baboons	*In vivo*	Whole blood	2.5–5	24, 48	Microarray	(*6*)
48	2.5–5 Gy/0.06								Microarray	
24	2.5–5 Gy/0.03								qRT-PCR	
48	2.5–5 Gy/0.03								qRT-PCR	
24	2–2.5 Gy/0.04	-	-	18 Baboons	*In vivo*	Whole blood	2–2.5	24	qRT-PCR	(*7*)
	4–5 Gy/0.16			18 Baboons,			4–5			
	2.5–5 Gy/0.33			15 Patients			2.5–5			
24	2.5 Gy/0.09	-	-	5 Baboons	*Ex vivo, in vitro*	Whole blood	2.5	24	qRT-PCR	(*7*)
	4 Gy/0.13			5 Baboons			4			
	1 Gy/0.43			5 Patients			1			
	2.5 Gy/0.2			5 Patients			2.5			
	4 Gy/0.17			5 Patients			4			
POU2AF1										
4	0.21	-	-	8 Patients	*In vivo*	Whole blood	1.25	4	Microarray	(*13*).
24	2.5–5 Gy/0.07	24–48	-	18 Baboons	*In vivo*	Whole blood	2.5–5	24, 48	Microarray	(*6*)
48	2.5–5 Gy/0.2								Microarray	
24	2.5–5 Gy/0.07								qRT-PCR	
48	2.5–5 Gy/0.2								qRT-PCR	
24	2–2.5 Gy/0.13	-	-	18 Baboons	*In vivo*	Whole blood	2–2.5	24	qRT-PCR	(*7*)
	4–5 Gy/0.29			18 Baboons			4–5			
	2.5–5 Gy/0.32			15 Patients			2.5–5			
24	2.5 Gy/0.11	-	-	5 Baboons	*Ex vivo, in vitro*	Whole blood	2.5	24	qRT-PCR	(*7*)
	4 Gy/0.1			5 Baboons			4			
	1 Gy/0.3			5 Patients			1			
	2.5 Gy/0.17			5 Patients			2.5			
	4 Gy/0.17			5 Patients			4			

Notes. This literature review (March 2020) focused only on the four genes (*FDXR*, *DDB2*, *POU2AF1* and *WNT3*) examined within our current study. The 24 and 48 h fold changes observed in previously published literature of our group (Port *et al*. 2016, 2018) are shown in the column entitled ''onset'' for better overview.

[a] Fold changes that represent estimated values drawn from cited figures.

some values in some individuals returning to normal or below normal at later times after highest dose (Supplementary Fig. S2; https://doi.org/10.1667/RADE-20-00126.1.S1). There was evidence of a dose dependency, with higher fold changes observed after 2–4 Gy relative to 0.5 Gy (Fig. 1 and Table 1).

In contrast to *FDXR* and *DDB2*, radiation caused a decrease in *POU2AF1* expression, with evidence of dose dependency; greater reductions were observed at the highest dose of 2 and 4 Gy and only a modest to non-significant decrease was observed after 0.5 Gy (Fig. 2). *POU2AF1* gene expression decreased roughly at twofold starting at 8 h and 4 h after 2 Gy and 4 Gy, respectively (Fig. 2 and Table 1). At the higher doses, the downregulation lasted for 24 h (5–10-fold downregulated, $P < 0.001$). Thereafter, levels returned back up to the normal range, reaching control values at 72 h (Fig. 2 and Table 1). Individual *POU2AF1* gene expression changes among the eight donors did not all follow the average course, with some individual samples showing inconsistent changes (Supplementary Fig. S3; https://doi.org/10.1667/RADE-20-00126.1.S1). A significant dose-response relationship was observed only after 24 h of culture time, while missing for the other time points, since 0 and 0.5 Gy on the one side and 2 and 4 Gy on the other side often revealed fold change of comparable degree (Fig. 3).

The expression of *WNT3* was decreased by higher dose of 2 and 4 Gy, reaching significance at 8 h and continuing to decline thereafter. At the 48-h time point, levels had fallen tenfold (Fig. 2 and Table 1). At the lowest dose tested, no consistent change could be detected, as indicated by the large variation in individual values (Fig. 2). Inter-individual variance was higher than for *FDXR* and *DDB2* (larger error bars in Fig. 2) and downregulation at later time points ranged between approximately tenfold (e.g., donor 1 and 3) or almost 100 fold (donor 8, Supplementary Fig. S4; https://doi.org/10.1667/RADE-20-00126.1.S1). Up to 12 h of cell culture time, no significant dose-response relationships could be observed. At later time points, linear-quadratic relationships were found, indicating a saturation of gene expression changes at $\geq$2 Gy (Fig. 3).

DISCUSSION

Radiological or nuclear scenarios demand an early and high-throughput diagnostic for prediction of the later occurring HARS, with its severity increasing from degrees 0–4 according to METREPOL. Recently, our laboratory identified a gene set that identified unexposed individuals from radiation-exposed individuals who developed H1 and H2–4 HARS severity (*6, 7*). Uniquely, our approach did not focus on dose estimation, but rather converted radiation-induced gene expression changes into medically relevant information. This smoothens the interface of radiobiology with clinical decisions about treatment with respect to later developing HARS severity. In our view, individual medical intervention can be improved beyond reliance on a difficult-to-interpret dose estimate.

Unfortunately, our previous studies on baboons and patients allowed only for measurements at 24 and/or 48 h postirradiation. Corresponding *in vitro* models in our previous work indicated similar *in vivo* as well as *in vitro* response to ionizing radiation for four genes, namely *FDXR*, *DDB2*, *WNT3* and *POU2AF1*. Taking advantage of the *in vitro* model, this allowed us to examine the beginning and the end of the HARS diagnostic window more carefully regarding these four genes. For the *in vitro* study, we have chosen 0.5, 2 and 4 Gy exposure patterns, corresponding to a gene expression response indicating 1st degree (0.5 Gy) or 2nd–4th-degree (2 and 4 Gy) HARS severity.

We have been able to confirm that the radiation-induced changes in the expression of candidate marker genes tested in our *in vitro* whole blood irradiation model are consistent with those we observe in blood obtained from irradiated baboons and leukemia patients receiving radiation treatment. For instance, earliest gene expression changes (*FDXR*) were detected at 4 h postirradiation, followed by 4 h (*DDB2* and *POU2AF1* at 4 Gy) and at 8 h (*WNT3*) (Fig. 2 and Table 1). These gene expression changes correlated both qualitatively and quantitatively with our previously reported studies on the peripheral blood of irradiated baboons and human leukemia patients receiving radiotherapy (*7*). This strengthens our interpretations using the *in vitro* model and indicates that the diagnostic window for prediction of the subsequent development of HARS requiring intervention may be available as early as 2–8 h, and until at least 72 h postirradiation. The wide range of doses causing significant changes in the gene expression suggests that the expression changes should be used with caution in assigning doses (Fig. 2 and Table 1).

The filter we have used as a cut-off for the definition of the diagnostic window required a significant twofold deregulation in gene expression to ensure robustness. Statistically significant differences are seen even earlier, e.g., at 1 h postirradiation for *FDXR* (2 Gy, $P < 0.001$) and *DDB2* (2 and 4 Gy with $P < 0.001$, $P = 0.015$, respectively). However, associated fold changes in gene expression $<$1.3 represent values lying within the methodological variance of the method and, therefore, would result in false-positive results.

Previously published work by several other groups supports our findings (Table 2). They report differential gene expression of *FDXR* and *DDB2* to occur 2–4 h and 3–4 h postirradiation *in vitro* and *in vivo*, respectively (*7, 9, 10, 12–18*). Corresponding published work regarding *POU2AF1* and *WNT3* is limited, and our own previous *in vivo* and *in vitro* studies are in agreement with the current data of this study (*6, 7, 9, 13*).

Results of our *in vitro* study furthermore indicate that the diagnostic window can be extended up to 72 h postirradiation regarding *FDXR* and *DDB2* because, for these genes, expression values are still significantly and several-fold upregulated (Fig. 2). A downregulation for *WNT3* at 72 h after 2–4 Gy dose is in support of the diagnostic window extension for *WNT3* as well; however, the underlying number of measurements decreased to 1–2 measurements per dose, thus reflecting the limits of our *in vitro* model. The same holds true for *POU2AF1* where control values were reached at 72 h postirradiation, which contrast to the downregulation of *POU2AF1* observed before (Fig. 2).

Clearly, our *in vitro* culture of white blood cells appears limited, as indicated by decreased WBCC starting at 12 h of culture time and a reduction of RNA yields, but this has only a minor impact on RNA quality and no effect on the housekeeping gene over the whole culture time (18S rRNA, Fig. 1). To adjust for the gene expression variance caused by the model, we had to use nonirradiated samples of the corresponding time point as the reference. Therefore, these data and the agreement with baboon and human data measured at 24 and 48 h postirradiation imply meaningful and trustworthy *in vitro* gene expression measurements that can be obtained over the first 48 h. Values measured after 72 h may have to be judged with caution, in particular regarding *POU2AF1* and *WNT3*.

Of note, gene expression measurements of individuals who are potentially exposed in a radiological or nuclear event do not require an *in vitro* cultivation of peripheral blood. Instead, peripheral blood will be drawn (earliest 2–4

h postirradiation) into commercially available blood tubes (e.g., PAXgene™ RNA blood tubes; BD Diagnostics, Hombrechtikon, Switzerland) for preserving RNA and inhibition of RNAses. No *in vitro* biased data would be generated.

Our experimental design captures inter-individual variance but not intra-individual variance since only one sample per individual was used for analysis. However, based on our experience, inter-individual variance largely covers expected intra-individual variance. Therefore, our interpretations will probably hold true even in the absence of these measurements. In addition, the results presented here are very much in agreement with many reports of other groups (as discussed above and shown in Table 2) which strengthens our interpretation.

In summary, our results indicate a diagnostic window for early HARS prediction based on gene expression changes starting as early as 2 h for most (75%) and at 4 h after exposure for all individuals examined and presumably lasting up to three days postirradiation.

SUPPLEMENTARY INFORMATION

Fig. S1. Differential gene expression of *FDXR* measured in the peripheral blood of eight healthy donors is shown over time after irradiation (cell culture). Fold changes are calculated with the nonirradiated samples of the same time point used as a reference to compensate for methodological variance. Symbols reflect the geometrical mean and error bars the min and max values of two technical replicate measurements.

Fig. S2. Differential gene expression of *DDB2* measured in the peripheral blood of eight healthy donors is shown over time after irradiation (cell culture). Fold changes are calculated with the nonirradiated samples of the same time point used as a reference to compensate for methodological variance. Symbols reflect the geometrical mean and error bars the min and max values of two technical replicate measurements.

Fig. S3. Differential gene expression of *POU2AF1* measured in the peripheral blood of eight healthy donors is shown over time after irradiation (cell culture). Fold changes are calculated with the nonirradiated samples of the same time point used as a reference to compensate for methodological variance. Symbols reflect the geometrical mean and error bars the min and max values of two technical replicate measurements.

Fig. S4. Differential gene expression of *WNT3* measured in the peripheral blood of eight healthy donors is shown over time after irradiation (cell culture). Fold changes are calculated with the nonirradiated samples of the same time point used as a reference to compensate for methodological variance. Symbols reflect the geometrical mean and error bars the min and max values of two technical replicate measurements.

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