DNA damage in human whole blood caused by radiopharmaceuticals evaluated by the comet assay

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Abstract

Radiopharmaceuticals used for diagnosis or therapy induce DNA strand breaks, which may be detectable by single-cell gel electrophoresis (called comet assay). Blood was taken from patients before and at different time points after treatment with radiopharmaceuticals; blood cells were investigated by the comet assay using the percentage of DNA in the tail as the critical parameter. Whereas [225Ac]Ac-prostate-specific membrane antigen (PSMA)-617 alpha therapy showed no difference relative to the blood sample taken before treatment, beta therapy with [177Lu]Lu-PSMA-617 3 h post-injection revealed a small but significant increase in DNA strand breaks. In blood of patients who underwent positron emission tomography (PET) with either [18F]2-fluor-2-deoxy-D-glucose (FDG) or [68Ga]Ga-PSMA-11, an increase of DNA migration determined by the comet assay was not found when analysed at different time points (2–70 min) after intravenous tracer injection. Human whole blood was incubated with the targeted clinically relevant therapeutic radiopharmaceuticals [225Ac]Ac-PSMA-617, [177Lu]Lu-PSMA-617 and [90Y]Y-DOTA(0)-Phe(1)-Tyr(3)-octreotide (DOTA-TOC) at different activity concentrations (kBq/ml) for 5 days and then analysed by the comet assay. DNA damage increased with higher concentrations of all radiolabeled compounds tested. [177Lu]Lu-PSMA-617 caused higher blood cell radiotoxicity than equal activity concentrations of [90Y]Y-DOTA-TOC. Likewise, whole human blood was exposed to the positron emitters [18F]FDG and [68Ga] Ga-PSMA-11 in vitro for 24 h with activity concentrations ranging between 5 and 40 MBq/ml. The same activity concentration dependent elevated DNA migration was observed for both compounds although decay energies are different. This study demonstrated that the amount of DNA damage detected by the comet assay in whole human blood is similar among different positron emitters and divergent by a factor of 200 between alpha particles and beta radiation.

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Introduction

The comet assay provides an excellent method for detecting initial DNA damage at the single-cell level (1). Under alkaline conditions, single-strand DNA breaks, double-strand DNA breaks and breaks resulting from abasic sites are detected. Recently, it has been demonstrated that whole human blood after storage at −80°C or −20°C showed no significant increase in DNA damage if aliquoted into small volumes (250 µl) (2). This storage procedure makes the blood samples suitable for direct application in the alkaline comet assay without further need for prior isolation of cells and is, therefore, almost predestined for their use in *in vivo* biomonitoring studies (3).

Radiopharmaceuticals commonly used for imaging and endoradiotherapy exert their genotoxic effect by ionising radiation, which depends on the amount of activity, physical radiation quality and cellular radionuclide deposit. So far the comet assay has only rarely been used to measure the genotoxic effect caused by radiopharmaceuticals, including [²²⁵Ac]Ac-DOTA(0)-Phe(1)-Tyr(3)-octreotide (DOTA-TOC) for alpha therapy and [¹⁷⁷Lu]Lu-DOTA-TOC for beta therapy (4–7), and to our knowledge positron-emitting radiotracers have not been tested before.

The aim of this study was to investigate whether the comet assay is able to detect DNA fragmentation in patients’ blood after administration of radiopharmaceuticals for targeted prostate cancer therapy ([²²⁵Ac]Ac-prostate-specific membrane antigen (PSMA)-617 and [¹⁷⁷Lu]Lu-PSMA-617) and radiotracers for cancer imaging ([¹⁸F]2-fluor-2-deoxy-D-glucose (FDG) and [⁶⁸Ga]Ga-PSMA-11).

Second, we used the comet assay to establish activity response relationships between radiopharmaceuticals and DNA damage caused in human whole blood by *in vitro* incubations in order to compare different radiation types and to estimate lower limits of detection.

Material and methods

Chemistry

[²²⁵Ac] was obtained by radiochemical extraction from [²²⁹Th] at the European Commission, Joint Centre, Directorate for Nuclear Safety and Security, Karlsruhe, Germany as described (8,9). The PSMA-617 precursor was obtained from ABX and labelled with [²²⁵Ac] as previously described (10). [¹⁷⁷Lu] labelling of PSMA-617 was performed as described (11). DOTA-TOC was labelled with [⁹⁰Y] (GE Healthcare, Braunschweig, Germany) as previously described (12). [¹⁸F]FDG was radiosynthesized at German Cancer Research Center (DKFZ) in a Nuclear Interface FDG synthesis module using [¹⁸F]fluoride obtained by nuclear reaction (¹⁰O(p,n)¹⁸F) from a Scanditronix MC32NI cyclotron. [⁶⁸Ga]Ga-PSMA-11 was radiosynthesized as previously described (13).

Blood collection and storage conditions

Human blood samples were drawn by venipuncture using a 21-gauge needle and syringe from antecubital veins into EDTA-containing Vacutainer tubes (3-ml Vacutainer, BD). Aliquots of 250 µl were stored at −80°C or −20°C as described (2). Blood samples from 10 patients receiving [²²⁵Ac]Ac-PSMA-617 (6–9 MBq, 100 kBq/kg b.w.) and from 10 patients receiving [¹⁷⁷Lu]Lu-PSMA-617 (standard treatment activity 6 GBq per patient) in clinical practice were taken just before (0 h) and at 3, 21–24 and 42–48 h after administration. Medical indication for blood samplings was the approximation of the treatment dosimetry, which depends on the amount of activity, physical radiation quality and cellular radionuclide deposit. So far the comet assay has only rarely been used to measure the genotoxic effect caused by radiopharmaceuticals, including [²²⁵Ac]Ac-DOTA(0)-Phe(1)-Tyr(3)-octreotide (DOTA-TOC) for alpha therapy and [¹⁷⁷Lu]Lu-DOTA-TOC for beta therapy (4–7), and to our knowledge positron-emitting radiotracers have not been tested before.

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Second, we used the comet assay to establish activity response relationships between radiopharmaceuticals and DNA damage caused in human whole blood by *in vitro* incubations in order to compare different radiation types and to estimate lower limits of detection.

**In vitro incubations**

To 45 µl of human whole blood from a healthy volunteer was added 5 µl of the radiopharmaceutical injection solution dissolved in Tris(hydroxymethyl)-aminomethan buffer at pH 7.4 and an activity concentration ranging between 1 and 50 kBq/ml for the alpha-particle-emitting radiotracer [²²⁵Ac]Ac-PSMA-617, between 10 and 400 kBq/ml for the beta-particle-emitting radiotracers [¹⁷⁷Lu]Lu-PSMA-617 and [⁹⁰Y]Y-DOTA-TOC and between 5 and 40 MBq/ml for the positron-emitting radiotracers [¹⁸F]FDG and [⁶⁸Ga]Ga-PSMA-11. Samples were mixed and left at −20°C for 5 days due to safety regulations when [²²⁵Ac]Ac-PSMA-617, [¹⁷⁷Lu]Lu-PSMA-617 and [⁹⁰Y]Y-DOTA-TOC were added. Mixtures of blood and [¹⁸F]FDG or [⁶⁸Ga]Ga-PSMA-11 were left for 24 h at −20°C. All incubations were performed in duplicate and at −20°C because small volumes of human whole blood when stored at −20°C are suitable for direct use in the alkaline comet assay (2).

**Comet assay**

Formation of DNA damage was assayed by the alkaline comet assay essentially as described by Mayer et al. (15). The test for sensitivity of the comet assay as used in this study is described in the supplement and indicated that DNA damage caused by gamma irradiation of 0.2 Gy is clearly detectable.

Whole blood from patients or healthy volunteers was quickly thawed and 3 µl of whole blood was mixed with low melting point agarose (0.6%; 500 µl) and 70 µl of the blood/agarose mixture were dispensed onto two areas of the CometSlides (Trevigen). Slides were placed on ice for 10 min and then transferred to pre-chilled lysis solution and incubated for 1 h at 4°C. In addition, mixtures from *in vitro* incubations were thawed and 5 µl were taken and mixed with agarose as mentioned before.

Subsequently, slides were transferred to a horizontal electrophoresis unit (High Throughput comet assay tank COMPAC-50, Cleaver Scientific Ltd) filled with alkaline electrophoresis buffer. After 20 min of DNA unwinding, electrophoresis (power pack CS300, Cleaver) was performed at 4°C at 0.8 V/cm for 20 min. Slides were rinsed for 20 min first in neutralisation buffer, then for 20 min in water and subsequently air dried and stained with 50 µl SYBR Green solution (Molecular Probes). Fifty-one comets per slide area were selected at random and evaluated by fluorescence microscopy and image software (Kinetic Imaging Komet 6.0 Andor Technology). The extent of DNA damage was measured quantitatively by the ‘parameter DNA intensity in tail’ (tail DNA in %). Results are expressed as mean of the medians from percentage of tail of at least four slide areas.

**Statistical aspects**

All *in vitro* incubations were performed in duplicate and each sample was analysed by the comet assay scoring 51 nucleoids per slide and 2 min, 10–13 min or 60–70 min after intravenous injection of [¹⁸F]FDG (206–254 MBq) and before or 60–70 min after intravenous injection of [⁶⁸Ga]Ga-PSMA-11 (108–262 MBq).

Time points for the blood sampling of patients were chosen according to clinical feasibility.

All patients published in this manuscript signed a written informed consent form for the purpose of anonymised evaluation and publication of their data. All reported investigations were conducted in accordance with the Helsinki Declaration and with our national regulations (German Pharmaceuticals Act, AMG §13 2b). This evaluation was approved by the ethics committee of the University of Heidelberg (S-321–2012).
two slides per incubation. Quantitative data were expressed as mean ± SD. If applicable, means were compared using Student’s t-test. P-values <0.05 were considered statistically significant.

All blood samples obtained from patients were measured on at least three different days by the comet assay scoring 51 nucleoids per slide and two slides per cryopreserved sample and analysed by repeated measures analysis of variance (ANOVA).

Blood from healthy volunteers was analysed along with the blood from patients in the same experiment resulting in a mean ± SD of 1.002 ± 0.105 tail DNA in %.

Results and discussion
Comet assay analysis with whole blood from patients receiving prostate cancer therapy
Blood from 10 patients receiving therapy with $^{225}$Ac-PSMA-617 (100 kBq/kg b.w.) was taken just before (0 h) and at 3, 21–24 and 42–48 h after administration by systemic injection. No difference in DNA migration was found between blood samples taken either before or after treatment with the alpha-particle-emitting radiotracer $^{221}$Ac-PSMA-617 when tested by the comet assay (Figure 1A). This might be due to the rapid elimination of PSMA-617 from blood (16); blood clearance was bi-phasic with half-lives of 4 and 90 h and the estimated blood/red marrow dose for this treatment was approximately 0.3 Sv RBE5. for $^{225}$Ac-PSMA-617 as reported previously (14). Considering a weighting factor of 5 for alpha particles, a relative biological effectiveness (RBE) of 0.3 Sv is unlikely to be sufficient to induce DNA strand breaks detectable in blood by the comet assay.

Ten patients receiving endoradiotherapy by systemic infusion of the beta-particle emitter-labelled tracer $^{177}$Lu-PSMA-617 (~6 GBq; $E_p$ ~ 0.49 MeV) were selected and blood was taken just before (0 h) or 3 and 42–48 h after treatment. Results of the comet assay showed a small but significant increase in DNA strand breaks at 3 h post-injection (factor 1.35; 95% confidence interval 1.16–1.54) but not at the later time point when analysed by repeated measures ANOVA (Figure 1B). This is in agreement with a report by Delker et al. (16) demonstrating that $^{177}$Lu-PSMA-617 does not bind to the surface of blood cells, is not taken up by passive diffusion and is rapidly distributed to the body water (approximately 22 l) 1 h post-injection. According to our previously reported dosimetry estimate, the blood/red marrow dose for these treatments was approximately 0.2 Gy (11), a dose level that is expected to induce detectable levels of DNA damage in the comet assay.

Comet assay analysis with whole blood from patients examined by PET
Blood from 10 patients (20–74 years old) undergoing PET was taken just before and after imaging with $^{18}$F-FDG (206–254 MBq). As shown (Figure 1C), no change in DNA damage assayed by the comet assay was detectable at 2 min, 10–13 min or 60–70 min after injection when compared to control or blood taken before injection. In contrast, May et al. (17) have shown that PET/CT with $^{18}$F-FDG induced DNA double-strand breaks in blood lymphocytes of patients detectable by the gamma-H2AX assay (18).

Figure 1. DNA damage (% Tail DNA; mean ± SD) in whole blood from 10 patients determined at different time points after application of (A) $^{225}$Ac-PSMA-617; (B) $^{177}$Lu-PSMA-617; (C) $^{18}$F-FDG and (D) $^{68}$Ga-PSMA-11. Error bars represent the mean of the medians obtained from the analysis of blood from 10 patients by the comet assay.
Blood from 10 patients (71–89 years old) undergoing PET was taken just before and 60–70 min after imaging with [68Ga] Ga-PSMA-11 (108–262 MBq). As found for the other positron-emitting radiotracer [18F]FDG, comet assay analysis of blood after [68Ga]Ga-PSMA-11 PET imaging did not result in elevated DNA migration (Figure 1D). This is in line with previous reports about this urea-based inhibitor of PSMA showing fast blood clearance in mice (19) and patients (20–22).

The comet assay measures single- and double-strand breaks in DNA at the level of individual cells. The biological consequences of such DNA damage entail the formation of mutations, cell death and the development of cancer. The level of DNA damage observed in the blood of patients receiving either alpha or beta therapy or patients examined by PET was for all time points investigated close to the background level found in the blood of a healthy volunteer suggesting no adverse effects on the patients.

In vitro incubations of radiopharmaceuticals with human whole blood

Blood taken from a healthy volunteer was exposed to [225Ac] Ac-PSMA-617 at −20°C in an activity concentration ranging between 1 and 50 kBq/ml. The samples were left for 5 days prior to being assayed by the comet assay to achieve a radioactivity concentration level below regulatory radiation safety requirements. As shown in Figure 2, an activity level dependent increase of DNA damage was found and under the conditions used, the lower limit of detection was estimated to be around 0.5 kBq/ml.

In vitro incubations with blood and the beta-particle-emitting radiotracers [177Lu]Lu-PSMA-617 and [90Y]Y-DOTA-TOC were also performed for 5 days at −20°C to make them comparable with the [225Ac]Ac-PSMA-617 incubations. At an activity concentration between 10 and 400 kBq/ml, both compounds showed an activity level dependent increase in DNA damage (Figure 3). However, [177Lu]Lu-PSMA-617 caused higher blood radiotoxicity detectable by the comet assay than [90Y]Y-DOTA-TOC, indicating that the low-energy electrons emitted by 177Lu (E_b=0.49 MeV) induced more DNA damage than the high-energy electrons emitted by 90Y (E_b=2.3 MeV). Comparable results have been reported by Wendisch et al. (23) when using the comet assay to compare DNA damage induced by the beta emitters 131I (E_b=0.807 MeV) and 188Re (E_b=2.12 MeV). The different ionisation density on the track of high- versus low-energy electrons is probably the main effect responsible for this observation.

Since the in vitro exposure with the PSMA radioligands [177Lu] Lu-PSMA-617 and [225Ac]Ac-PSMA-617 was performed under identical conditions and both radionuclides have similar physical half-lives (225Ac: 9.9 d; 177Lu: 6.73 d) we compared the DNA damage induction of alpha and beta particles in human whole blood. A DNA damage of around 6% Tail DNA was caused by 2 kBq/ml of [225Ac]Ac-PSMA-617, whereas the same amount of DNA damage is found with a 200-fold higher activity concentration of [177Lu]Lu-PSMA-617. Also, using the comet assay, Graf et al. (4) reported a 700-fold difference in activity amount resulting in the same DNA damage between the therapeutic radionuclides 225Ac and 177Lu when tested as radiolabelled somatostatin receptor ligands ([225Ac] Ac-DOTA-TOC and [177Lu]Lu-DOTA-TOC) in a cell line characterised by strong somatostatin receptor expression. Using the same activity amounts, the difference between 225Ac and 177Lu of only...
200-fold higher blood cell toxicity for $^{225}$Ac versus 700-fold higher damage in cancer cells for $^{222}$Ac might be explained by the subcellular micro-biodistribution of the respective radiopharmaceutical; radioactive somatostatin analogues are internalised bringing the decay event closer to the cell nucleus, in contrast radioactive PSMA-ligands show no binding to blood cells (16). This micro-dosimetry effect is more pronounced in short-range alpha radiation. Both results highlight the strong genotoxic effect of alpha particles emitted by $^{225}$Ac.

DNA damage induced by the positron emitters $[^{18}$F$]FDG$ and $[^{68}$Ga$]Ga$-PSMA-11 was investigated also by in vitro incubations with whole human blood. As shown in Figure 4 both compounds showed the same activity level dependent increase in DNA migration assayed by the comet assay although decay energies are quite different ($^{18}$F $E_{b+} = 643 \text{ keV}$; $^{68}$Ga $E_{b+} = 1.9 \text{ MeV}$).

Conclusions

This study demonstrates that the level of DNA damage detected by the comet assay in whole human blood is similar among the positron emitters $^{18}$F and $^{68}$Ga and different by a factor of 200 between alpha particle radiation with $^{225}$Ac and beta radiation with $^{177}$Lu. The level of DNA damage observed in the blood of patients receiving either alpha or beta therapy or patients examined by PET was close to the background level found in the blood of a healthy volunteer suggesting no adverse effects on the patients.

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Figure 4. DNA damage (% Tail DNA; mean ± SD) in whole human blood determined after incubation for 24 h at −20°C with different activity amounts of $[^{18}$F$]FDG$ and $[^{68}$Ga$]Ga$-PSMA-11. Blood was taken from a healthy volunteer. The results represent the means ± SD of two individual experiments scoring 51 nucleoids per slide and two slides per incubation on the same day.
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