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The Gottingen minipig is a model of the hematopoietic acute radiation syndrome: G-CSF stimulates hematopoiesis and enhances survival from lethal total-body gamma-irradiation

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Abstract

Purpose—We are characterizing the Gottingen minipig as an additional large animal model for advanced drug testing for the Acute Radiation Syndrome (ARS), to enhance discovery and development of novel radiation countermeasures. Among the advantages provided by this model, the similarities to human hematological parameters and dynamics of cell loss/recovery following

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Conflict of Interest Notification

No potential conflicts of interest exist.

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irradiation provide a convenient means to compare efficacy of drugs known to affect bone marrow cellularity and hematopoiesis.

Methods and Materials—Male Gottingen minipigs, 4–5 months old and weighing 9–11 kg were used for this study. We tested the standard off-label treatment for ARS, rhG-CSF (Neupogen®, 10 µg/kg/day for 17 days), at the estimated LD70/30 total-body gamma-irradiation (TBI) radiation dose for the hematopoietic syndrome, starting 24 hours after irradiation.

Results—Results indicate G-CSF enhanced survival, stimulated recovery from neutropenia, and induced mobilization of hematopoietic progenitor cells. In addition, administration of G-CSF resulted in maturation of monocytes/macrophages.

Conclusion—These results support continuing efforts toward validation of the minipig as a large animal model for advanced testing of radiation countermeasures and characterization of the pathophysiology of ARS, and suggest that the efficacy of G-CSF in improving survival after total body irradiation may involve mechanisms other than increasing numbers of circulating granulocytes.

Introduction

Beneficial effects of colony stimulating factors (CSFs) for victims of the hematopoietic syndrome are based on enhancement of neutrophil recovery. A global consensus on the administration of CSFs for the management of the hematopoietic sub-syndrome of the ARS emerged from a World Health Organization meeting in 2009 (1) based on the survival of fourteen out fifteen victims exposed to doses less than 5 Gy and treated with CSFs (G-CSF and GM-CSF).

The value of the consensus is hampered by the lack of human control groups. On the other hand, well-controlled animal trials on rodents, canines and nonhuman primates (NHPs) have provided evidence of the efficacy of CSFs in reducing lethality and facilitating marrow recovery following exposure to whole body irradiation. For canines, improved survival, hematopoietic recovery and decreases in duration and severity of neutropenia were observed following administration of CSFs in the presence of supportive care, at sub-lethal and lethal doses of whole body irradiation with gamma rays and mixed fission neutron:gamma rays (2–4, and list of references in 3). In rhesus monkeys, administration of G-CSF reduced duration of neutropenia and thrombocytopenia (5–6) similarly to what was observed for GM-CSF.

Only NHPs and canines have been well-described in terms of response to acute radiation exposure;. However, a variety of animal models would facilitate demonstration of absorption, distribution, metabolism, excretion and pharmacodynamics as close as possible to human for each drug candidate. We are characterizing the minipig as an additional large animal model for advanced drug testing for ARS (7–9). We have shown that the minipig is a suitable model for longitudinal blood sampling in irradiated, immunocompromised animals, and that the hematopoietic sub-syndrome of the ARS closely mimics that in humans in terms of clinical signs, symptoms and kinetics of blood cell depletion and recovery. Additional efforts are required to prove the validity of this model for the study of the ARS and to demonstrate equivalence to other animal models. Here, we describe the effect of G-CSF administered to Gottingen minipigs gamma-irradiated at the estimated LD70/30 on hematopoietic recovery, survival, clonogenic activity of progenitor stem cells from peripheral blood, cell cycle, peripheral blood mononuclear cells (PBMC) phenotypic changes, and microscopic pathology. Our results indicate the role of G-CSF in the minipig is equivalent to that observed in NHPs and in canines, and support the continuing efforts to address the validity of this model for the study of the hematopoietic sub-syndrome of the ARS and for the advanced development of radiation countermeasures (10). G-CSF was found to remove radiation-induced cell-cycle block in CFU colonies from hematopoietic

progenitor stem cells, and to induce maturation of monocytes/granulocytes, as occurs in humans.

We conclude the Gottingen minipig represents a suitable large animal model to study the effect of radiation and to understand the basic pathophysiology of ARS.

Materials and Methods

Animals

Thirty-two male Gottingen minipigs (4–5 months old, 9–12 kg) were purchased from Marshall Bioresources (North Rose, NY). All animal procedures were performed in accordance with protocols approved by the to Armed Forces Radiobiology Research Institute (AFRRI) Institutional Animal Care and Use Committee. Minipigs were fed twice daily (Harlan Teklad Minipig diet 8753, Madison, WI) according to individual weights and provider recommendations. Water was provided *ad libitum*. A vascular access port (VAP) was implanted two weeks after arrival of the animals, to facilitate blood sampling (8).

Drug administration

Neupogen® (G-CSF) was administered daily, s.c., at a concentration of 10 µg/kg/day, starting 24 hours (+/- 1 hour) after irradiation and continuing for 17 days, until the absolute neutrophil count (ANC) started to recover.

Irradiation and blood sampling

Animals were irradiated at 1.78 Gy with ⁶⁰Co (0.6 Gy/min, TBI) or sham-irradiated. after anesthetization with Telazol® (6–8 mg/kg im) and administration of atropine (0.05 mg/kg im) to decrease salivary secretions. Dose rates in the cobalt facility were determined using cylindrical water-filled Plexiglas® phantoms appropriate for the minipigs and the alanine-EPR system. The dose was to the midline tissue at the widest point of the core, with the animal laying in the sling and the width of the animal measured with a caliper. Real-time dosimetry was performed during the minipig irradiations with an ionization chamber as a quality control check. All procedures for irradiation and blood sampling are described in detail elsewhere (8). Blood samples were obtained from the VAP, collected in EDTA tubes and immediately processed for complete blood counts with differentials (CBC/diffs) using the ADVIA 2120 (Siemens Medical Solutions Diagnostics, Ireland). Blood samples irradiated *in vitro* for colony-forming unit assays were transferred to 15 mL conical tubes, transported on ice to the cobalt facility, irradiated (bilateral, 0.6 Gy/min, ⁶⁰Co) at the doses indicated, and placed back on ice until peripheral blood mononuclear cell (PBMC) isolation.

Necropsy and Histology

Full necropsies were performed on all animals and a full complement of tissues were collected. Sections were trimmed at 5–6 µm thickness, stained with hematoxylin and eosin, and evaluated by a board-certified veterinary pathologist.

Flow cytometry: whole blood (CD163+CD61) and PBMCs (Hoechst staining for side population)

Whole blood (20 µL) was incubated for 30 min at room temperature with 5 µL each of anti-CD163-RPE (AbD Serotec, MCA2311PE) and anti-CD61-FITC (AbDSerotec, MCA2263F). After staining, samples were immediately fixed and lysed and analyzed on a Guava flow cytometer.

Colony-forming units and cell cycle assay

For experiments requiring *in vitro* incubation with G-CSF, isolated PBMC samples were incubated with the indicated concentrations of G-CSF overnight at room temperature before seeding on methylcellulose medium (MethoCult) (5×10^5 PBMCs/plate). Colonies were counted 14 days after seeding.

C-reactive protein

Concentrations of C-reactive protein (CRP) in blood plasma samples were assessed using a commercially available porcine-specific ELISA kit (Innovative Research, RE-5BCRP), according to the manufacturer's instructions. Single-use plasma aliquots were thawed on ice, diluted 1:2000 and measured in duplicate using a plate reader (SpectraMax M5, Molecular Devices) set at an absorbance of 450 nm.

Statistical methods

Basic statistical analysis and two-sided Student's T-tests were done in Excel.

RESULTS

Effect of G-CSF in healthy, sham-irradiated pigs

The effects of Neupogen® were studied in eight animals receiving 10 µg/kg/day, s.c., starting 24 hours after sham-irradiation and continuing for 17 consecutive days. The control group was given an equivalent volume of sterile saline solution. In the drug-treated group, production of WBC increased about 3 fold within 24 hours, and reached a peak around days 10–17; counts returned to normal values within 7 days. WBC increases were due mainly to neutrophils, whose kinetics and magnitude of increase paralleled those of the WBC (Figure 1). Basophils were induced at day 7–17 ($p < 0.001$, Student's T-test), monocytes at day 7–10 ($p < 0.001$, Student's T-test); lymphocytes, and eosinophils were marginally induced; while effects on erythrocytes, platelets, hemoglobin and HCT were negligible (data not shown).

Effect of G-CSF in irradiated pigs—To evaluate the efficacy of G-CSF to improve survival, bone marrow recovery, release of hematopoietic progenitor cells and reduction of cytopenias in irradiated animals, a total of sixteen minipigs were irradiated at the predicted LD70/30 (TBI, Co-60, 0.1 Gy/min) and given G-CSF ($n=8$) or saline ($n=8$) as described above. The predicted LD70/30 was calculated from our previous probit curve (7). No supportive care was provided, in order to isolate the effects of G-CSF treatment. Lethality in the saline-treated group was 87.5%; G-CSF was effective in enhancing survival of animals irradiated by 37.5% (Figure 2). All surviving animals were euthanized at 30 days after irradiation.

As expected, G-CSF induced production of neutrophils and hastened recovery. For saline-treated animals, neutropenia ($< 0.5 \times 10^3$ ANC μL^{-1}) occurred at days 14–20; the mean nadir was 81 ± 18 s.e.m. and the duration was greater than 2 weeks. Cell nadirs were calculated as the mean of the cell nadir of the individual animals in each cohort. Recovery started 3 weeks after irradiation. For G-CSF-treated animals, ANC elevations were observed in irradiated animals 24 hours after drug administration. Neutropenia occurred at days 10–14; the nadir was 691 ± 363 s.e.m. The nadir was not significantly different from the saline group ($0=0.07$). Recovery started at day 14–17. Surviving IR+G-CSF animals did not manifest febrile neutropenia (FN); 3 out of 4 non-surviving G-CSF treated animals displayed FN, starting at day 10–14 and lasting until the animal crashed (and the temperature started to fall quickly). Platelet recovery was faster in the G-CSF group, starting at day 17, but the nadir was not affected ($p = 0.19$). Levels were above the threshold for

thrombocytopenia ($<20,000/\mu\text{L}$) at day 20. In the saline controls, recovery started at day 27 and remained below thrombocytopenic levels for the rest of the study (30 days) (Figure 3).

Besides increasing the number of circulating immune cells, myeloid CSFs enhance innate cell immunological features by increasing chemotaxis, maturation, phagocytosis, adhesion and other functions. We examined CD61 and CD163, markers of adhesion and maturation of monocytes/macrophages, respectively, in individual whole blood samples taken before and after irradiation. Treatment with G-CSF induced expression of CD163 already at 24 hours after *in vivo* administration in all animals (2 days after irradiation) (Figure 4); expression persisted for several days beyond the last drug administration in surviving animals (data not shown). When gating on CD163+ cells to assess presence of CD61 adhesion molecules, we found that animals with poor prognosis ($n=4$) displayed lower expression of CD61 with respect to animals that survived 30 days ($n=4$) (Figure 4; $p<0.05$). These observations were confirmed over 4 independent *in vivo* experiments, suggesting that maturation of monocytes/macrophages may play an important role in the resolution of ARS.

Peripheral blood-derived clonogenic activity—We assessed clonogenicity of hematopoietic progenitors from PBMCs irradiated *ex vivo* and *in vivo* (Figure 5, panel A). Irradiation decreased both proliferation and differentiation potential of hematopoietic stem cells; loss of colonies was dose- and time-dependent. G-CSF stimulated *in vitro* recovery of clonogenic/differentiation potential (Figure 5, panel B). Administered 24 hours following irradiation at the LD70/30, G-CSF stimulated release of myeloid progenitors and favored recovery already at day 2 after exposure. By day 7, almost no colonies could be recovered from the saline control animal group, while a significant number of progenitors were present in the peripheral blood of G-CSF-treated animals (Figure 5, panel C).

Pathology—Bone marrow, lungs, heart and liver were less affected in the G-CSF-treated group (Table 1). Bone marrow depletion was more evident in saline-treated as compared to G-CSF-treated pigs that were more likely to have panhypoplastic bone marrow.

Lymphoid atrophy within lymph nodes and gastrointestinal- and bronchial-associated lymphoid tissue occurred in both treatment groups, but was more common in saline-treated pigs. Panhypoplastic bone marrow consisted of generally less than 25% of the marrow space containing hematopoietic cells, while depleted bone marrow was essentially devoid of hematopoietic cells with marrow spaces containing primarily adipose tissue and vascular sinuses. Microscopic evidence of hemorrhage within lymph nodes, pancreas, urinary bladder and gastrointestinal tract were similar between the treatment groups. Approximately half of both treatment groups had pneumonia with large colonies of bacteria.

DISCUSSION

The minipig is emerging as an alternative large animal model to characterize ARS (7, 11–12), and we are in the process of validating the model. In this study, we addressed two fundamental questions related to the validity of this model: reproducibility of the survival curve at hematopoietic doses, and comparison of the effects of G-CSF between minipigs, humans, NHP and dogs. The results presented here show that we are within 20% of our published relationship of dose versus mortality (7), which is considered an acceptable range in other models (mice, NHP). Additional testing is required to confirm this range in mortality; however we expect that higher numbers of animals/group will reduce the variability. Administration of G-CSF in irradiated minipigs provides the same beneficial effects observed in other species. In addition, we found that treatment with G-CSF impacted monocyte maturation.

A number of radiation countermeasures induce G-CSF and enhance survival in a G-CSF-dependent manner (13). In humans, CSFs accelerate neutrophil recovery by 3–6 days after myeloablative therapies. A study by REAC/TS Radiation Emergency Assistance Center/Training Site (14) reports that out of twenty-eight victims, twenty-five showed signs of faster neutrophil recovery following administration of CSFs with or without interleukin-3. The effect of G-CSF on survival and hematopoietic recovery in NHPs and canines in the presence of supportive care has been extensively studied. G-CSF improved survival in both species. In NHPs, a 38% increase in survival following a 50% lethal dose of total body irradiation was observed (6). In dogs, G-CSF improved survival by 40–80% in the lethal dose range 3.6 – 5.0 Gy and by 40% after irradiation with 2.3 Gy mixed fission neutron-gamma irradiation (3–4). Increases in survival were accompanied by stimulation of bone marrow-derived clonogenic activity in both NHPs and canines, and reduction of the duration of cytopenia and time to recovery for both neutrophils and platelets (2–6, 15–16); significant modulation of the neutrophil nadir was observed for canines irradiated at 3.5 Gy (3), but not in NHPs (6). It is interesting that human G-CSF has similar hematopoietic effects in multiple species (mouse, canine, swine, NHP, human), at roughly similar doses. However, the precise comparative absorption, distribution, metabolism, excretion and pharmacodynamics need to be worked out to determine how differences may affect use of G-CSF as a positive control in advanced development of novel countermeasure candidates.

The current study was carried on in the absence of supportive care, to understand the basic pathophysiology of ARS without potential confounding factors introduced by blood, hydration, antibiotics, anti-inflammatory treatments, etc. Animals were irradiated at the LD70/30; results confirmed the reproducibility of the survival curves over a period of 5 years. Daily administration of G-CSF starting 24 hours after exposure improved survival over controls by 37.5%, and reduced duration of neutropenia and thrombocytopenia. Microscopically, though both treatment groups had significant loss of marrow hematopoietic cells, G-CSF-treated pigs had less depletion. Duration of thrombocytopenia and neutropenia as well as time to recovery was decreased by G-CSF administration. Following irradiation, administration of G-CSF increased the number of circulating progenitors and proliferation and differentiation potential. After G-CSF administration, PBMCs express phenotypic changes that result in an activation status, with increased adherence, oxidative burst and phagocytosis. One example is the induction of CD163 on monocytes and tissue-resident macrophages in humans following stimulation with GM-CSF and M-CSF. CD163 is a marker of maturation, and its expression pattern appears to reflect a functional role in the anti-inflammatory response of monocytes (17). Impairment in monocyte maturation and function of differentiated dendritic cells has been observed after irradiation in humans. To further confirm that the minipig represents an appropriate model for human treatment, we tested expression of CD163. Porcine CD163 has a sequence similar to that of human CD163 (18). Differences in CD163 expression are associated with differences in the pattern of cytokine production and maturation stage. CD163+ cells are considered resident monocytes/macrophages (19). Maturation of CD163+ cells is associated with a progressive increase in the expression of adhesion molecules, including CD61 (19). We found that G-CSF induced expression of CD163+ monocytes/macrophages; however, only co-expression of CD163 and CD61 was predictive of survival in irradiated animals. Correlation of CD163+ CD61+ cells with survival, as shown here, suggests that expression of adhesion molecules on mature monocytes/macrophages is required for trafficking and homing to inflamed tissues, as already observed for hematopoietic stem-progenitor cell engraftment (20).

CONCLUSION

In conclusion, the effects in other species of G-CSF on survival, duration of cytopenias and time to recovery, mobilization of hematopoietic progenitor stem cells and recovery of the

bone marrow are consistent with what we observed in a minipig model of bone marrow syndrome. In addition, we extended the similarities of G-CSF's mechanism of action between humans and pigs by confirming in the swine model the cell-cycle priming effect and the induction of monocyte/macrophage maturation. These data confirm the suitability of the minipig as a large animal model for testing of radiation countermeasures. Longer observation periods extending to 60 days and the use of supportive care should be considered when testing MCMs for H-ARS, to account for potential delayed mortality, dissociated hematological recovery and secondary relapse.

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Summary

The minipig is emerging as a large animal model to characterize the Acute Radiation Syndrome. We addressed reproducibility of the survival curve for drug testing, and comparison of the effects of G-CSF in the minipig with humans and other large animal models. This is the first study showing efficacy of G-CSF in irradiated minipigs, and demonstrates the validity of the model for countermeasure drug testing.

Figure 1. Effect of G-CSF administration on peripheral blood WBC and neutrophil counts in sham-irradiated minipigs

Administration of G-CSF or saline was initiated 24 hours after irradiation. Symbols represent single animals (white: saline treated, black: G-CSF treated), lines represent averages of groups. N = 8 animals/group.

Figure 2. Survival curve after estimated LD70/30 TBI dose (Cobalt-60, 0.6 Gy/min), calculated from our previous probit curve, in the absence of supportive care, in minipigs receiving G-CSF or saline starting 24 hours after irradiation. Survival in the G-CSF-treated group (50%) was 37.5% higher than in the vehicle-treated group (12.5%). N = 8 animals/group.

Figure 3. Effect of G-CSF administration on peripheral blood neutrophil and platelet counts in irradiated minipigs

Administration of G-CSF or saline was initiated 24 hours after irradiation. Counts represent means $\pm$ s.e.m. Dashed arrows indicate threshold for neutropenia or thrombocytopenia. N = 8 animals/group.

Figure 4. G-CSF stimulates maturation and adhesion of monocytes

Animals were irradiated at day 0. Twenty-four hours later, G-CSF treatment was started. Whole blood was taken at the time indicated, and immediately stained. CD163+ cells were gated and analyzed for expression of CD61 antigen and expressed as percentage of double positive cells.

Figure 5. Irradiation causes loss of hematopoietic progenitor cells from peripheral blood. Administration of G-CSF stimulates proliferation of progenitors

Colonies from PBMCs exposed *ex vivo* to various radiation doses or obtained at different time points after 1.8 Gy *in vivo* irradiation are shown (Panel A). G-CSF stimulated proliferation of hematopoietic progenitors (Panel B). *In vivo*, G-CSF stimulated the release and proliferation of hematopoietic progenitors (Panel C).

Table 1

Histopathological findings in minipigs irradiated at the LD70/30 and treated with either saline or G-CSF

Treatment	Irradiated + saline (n=8)	Irradiated + G-CSF (n=8)
Lungs	Severe pulmonary hemorrhage, fibrin, edema (71%)	Moderate to severe pulmonary hemorrhage, edema (43%)
Heart	Mild to severe cardiac hemorrhage (86%)	Mild to moderate cardiac hemorrhages (29%)
Liver	Moderate vacuolar change within hepatocytes (57%)	Moderate vacuolar change within hepatocytes (14%)
Bone marrow	Severe depletion	Severe pan-hypoplasia
Spleen	Mild to moderate lymphoid atrophy (100%)	----