Cytokine Treatment Improves Parenchymal and Vascular Damage of Salivary Glands after Irradiation

Isabelle M.A. Lombaert,1,2 Jeanette F. Brunsting,1 Pieter K. Wierenga,2 Harm H. Kampinga,1 Gerald de Haan,2 and Robert P. Coppes1,3

Abstract Purpose: During radiotherapy for head and neck cancer, co-irradiation (IR) of salivary glands results in acute and often lifelong hyposalivation. Recently, we showed that bone marrow-derived cells (BMC) can partially facilitate postirradiation regeneration of the mouse submandibular gland. In this study, we investigate whether optimized mobilization of BMCs can further facilitate regeneration of radiation-damaged salivary glands.

Experimental Design: Salivary glands of mice reconstituted with eGFP+ bone marrow cells were irradiated with a single dose of 15 Gy. One month later, BMCs were mobilized using granulocyte colony-stimulating factor (G-CSF) or the combination of FMS-like tyrosine kinase-3 ligand, stem cell factor, and G-CSF (termed F/S/G) as mobilizing agents. Salivary gland function and morphology were evaluated at 90 days post-IR by measuring the saliva flow rate, the number of acinar cells, and the functionality of the vasculature.

Results: Compared with G-CSF alone, the combined F/S/G treatment mobilized a 10-fold higher number and different types of BMCs to the bloodstream and increased the number of eGFP+ cells in the irradiated submandibular gland from 49% to 65%. Both treatments reduced radiation-induced hyposalivation from almost nothing in the untreated group to ~20% of normal amount. Surprisingly, however, F/S/G treatment resulted in significant less damage to submandibular blood vessels and induced BMC-derived neovascularization.

Conclusions: Post-IR F/S/G treatment facilitates regeneration of the submandibular gland and ameliorates vascular damage. The latter is partly due to BMCs differentiating in vascular cells but is likely also result from direct stimulation of existing blood vessel cells.

Radiation-induced damage to normal tissues may result in organ dysfunction, limit the optimal treatment dose, and/or cause a reduction in the quality of life of a patient post-treatment. Radiotherapy of head and neck cancer patients, which often involves co-irradiation (IR) of the salivary glands, induces hyposalivation with concomitant symptoms like oral dryness, dental caries, oral infections, difficulties in speech, and food mastication (1, 2). Some therapies to treat salivary gland damage, such as saliva substitutes, are available (reviewed refs. 1, 2) but are often insufficient or not applicable to all patients. Although intensity-modulated radiation therapy enables a reduction of the dose delivered to the salivary gland, many patients still suffer from salivary gland dysfunction. Therefore, new treatments that could reduce post-IR salivary gland damage would be of major benefit. One approach to achieve this may be the use of stem cells to allow regeneration of the radiation-damaged tissue.

Radiation-induced sterilization of salivary stem cells precludes the replacement of functional differentiated saliva-producing acinar cells. In addition, radiation affects the vasculature (3–5), which is very important for the proper functioning of the salivary glands. Capillary endothelial cell swelling and increased capillary permeability, resulting in the detachment of endothelial cells from the basal lamina, cell pyknosis, thrombosis, and even loss of entire capillary segments (6), are typical signs of radiation-induced damage to the vasculature. Beyond reduced capillary density, dilation of larger blood vessels can induce ischemia and secondary tissue function loss (7).

We recently showed that treatment with granulocyte colony-stimulating factor (G-CSF), inducing mobilization of bone marrow-derived cells (BMC) to the bloodstream, resulted in the abundant presence of BMCS in irradiated salivary glands and improved morphology and function (8). Similar observations have been made in tissues like skin (9), lung (10), kidney
Translational Relevance

During radiotherapy of head and neck cancers, co-IR of salivary glands may lead to long-term hyposalivation resulting in xerostomia (dry mouth syndrome), severely compromising the patient’s quality of life. Currently, no adequate treatments are available. In the present study, we show that bone marrow cells mobilized using different cytokines facilitate the repair of saliva tissue after IR. It improves morphology and function, as both the number of saliva-producing cells increase, which is accompanied by enhanced vascularization. As for affected patients a very small increase in saliva production is of substantial benefit, our data are of great clinical relevance. Furthermore, improved vascularization as we have observed in the salivary glands could benefit many other radiation-damaged tissues that suffer from vascular loss, including heart, kidney, and bone. G-CSF is already commonly used for hematopoietic diseases and is currently investigated for angiomyogenesis in myocardial infarction. G-CSF in humans is a very good cell-mobilizing agent and it may not be necessary to use a combination of cytokines. Our protocol could be readily adapted for a clinical trial in patients.

Materials and Methods

Animals. Female C57BL/6 mice, 8 to 12 weeks old, were purchased from Harlan and used as recipient mice in a sex-mismatched bone marrow transplantation setting. Donor mice were GFP+ male C57BL/6-TgN (ArtheGFP) bred in the animal facility of the University Medical Center Groningen. All mice were kept under clean conventional conditions and fed ad libitum with food pellets (RMH-B; Hope Farms) and acidified tap water (pH 2.8). All experiments were approved by the Animal Ethical Committee on Animal Testing of the University of Groningen.

Bone marrow transplantation protocol. Mice were splenectomized under halothane/O2 anesthesia and allowed to recover for at least 2 weeks. Splenectomized female mice were given 9.5 Gy total body IR of X-rays (Philips CMG 41 X, 200 kV, 10 mA, 5 Gy/min) and were transplanted with 3×106 male whole bone marrow cells from GFP+ transgenic mice by intravenous injection (Fig. 1A). During the total body IR, the salivary glands were shielded with a 12×50×3 mm lead plate and received <5% of the dose delivered to the rest of the body. Bone marrow cells were obtained by flushing the femoral content with Iscove’s modified Dulbecco’s medium (Life Technologies). Using the FACSCalibur flow cytometer (Becton Dickinson), chimerism in peripheral blood samples was evaluated 8 weeks later (Fig. 1A). Only mice with >60% chimerism were used for further experiments.

Gland IR. Eight weeks after eGFP bone marrow transplantation, the salivary glands of the chimeric mice were locally irradiated with a single dose of 15 Gy (as described above), now shielding the rest of the body with 3 mm lead (Fig. 1A). This dose is known to induce sufficient damage without compromising the general health of the animals and is biologically equivalent to a clinical relevant fractionated dose of 16×2 Gy (33). The extra dose delivered to the bone marrow will be <5% (8, 34).

Determination of optimal progenitor mobilization protocol. To determine the best protocol to mobilize BM BMCs to the blood, normal mice were treated with three different cytokines or combination thereof. BM BMCs were mobilized by two subcutaneous injections of 25 μg PEG-rHu-G-CSF (Amgen) given 3 days apart (8), 10 μg Flt-3L (Amgen), or 2.5 μg SCF (Amgen) via a subcutaneously implanted osmotic pump (Alzet) for 7 constitutive days. A combined treatment protocol consists of PEG-rHu-G-CSF in combination with Flt-3L and SCF, all as described above, starting on the day of the first G-CSF injection. The combined treatment with Flt-3L, SCF, and G-CSF is depicted as F/S/G. Blood samples were taken from mice at days 3, 6, 10, and 14 after the first injection day. Hematopoietic progenitor cells were assayed as described earlier (35). Briefly, cells were plated in α-medium (Stem Cell Technologies) containing 0.8% methylcellulose (Fluka), 30% FCS (Life Technologies), and 105 nucleated cells/mL. Colony growth was stimulated by granulocyte-macrophage CSF (10 ng/mL) and SCF (100 ng/mL). Cultures were plated in 35 mm polystyrene culture dishes (Falcon, Becton Dickinson) and grown at 37°C in a 5% CO2 humidified atmosphere. Colonies (>50 cells) were scored after 7 days of culture.

More primitive cell subsets were assayed by the cobblestone area-forming cell (CAFC) assay (35). This assay consists of a confluent FBMD-1 cell culture in 96-well plates (Costar) overlaid with mobilized peripheral blood cells in a limiting dilution setup. Eight dilutions 2-fold apart were used with 10 replicate wells per dilution. The cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 20% horse serum (Life Technologies) at 33°C in a 10% CO2 humidified atmosphere with a half-volume medium change every week. The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells beneath the stromal layer was determined 4 to 5 weeks after initiating the culture. Cobblestone area frequency was calculated using Poisson statistics.

At 30 days post-gland IR (Fig. 1A), bone marrow cells were mobilized with PEG-rHu-G-CSF or F/S/G as described above. Untreated
normal mice and normal mice with irradiated salivary glands served as controls.

Saliva collection. Whole saliva flow rate was determined 90 days post-gland IR (Fig. 1A). The animals were placed in a restraining device (36) after pilocarpine injection (2 mg/kg subcutaneous). Saliva was collected for 15 min and determined gravimetrically, assuming a density of 1 g/mL for saliva (8).

Immunohistochemistry. At 90 days post-IR of the salivary glands, animals were sacrificed for immunohistologic analysis. The submandibular glands were extirpated and incubated for 29 h at 4°C in 4% buffered formaldehyde. Following dehydration, the tissue was embedded in paraffin. Sections (5 μm) were analyzed for the presence of eGFP using a confocal scanning laser microscopy (Leica TCS SP2). Counterstaining was done with 4',6-diamidino-2-phenylindole.

CD31 expression was evaluated by overnight staining with anti-CD31 antibodies (BD Pharmingen; 1:100) following 0.25% trypsin pretreatment (Life Technologies/Invitrogen) at 37°C. The prevalence of blood vessels, labeling using antinecrotic antibodies (DAKO) and an avidin-biotin-horseradish peroxidase complex (Vector Elite Avidin-Biotin Complex kit). To investigate the prevalence of blood vessels, labeling using an anti-Flt3 R antibody (Cell Signaling Technologies), anti-endothelial nitric oxide synthase (eNOS; Santa Cruz Biotechnology) following citrate or trypsin pretreatment was done. Coexpression of CD31 with eGFP was evaluated by labeling the tissue sections with anti-CD31 and subsequent anti-rat rabbit anti-rat type 3 antibody to examine the surface area occupied by each gland. Using the AnalySIS program (Olympus, Soft Imaging System), the surface area occupied by each gland was enumerated and converted into a percentage per 100 mm2 gland surface. The number of capillaries per 180,000 mm2 measured gland area was enumerated and converted into a percentage per 100 μm2. Vascular changes in endoglin, PCNA, and eNOS expression were evaluated using a semiquantitative scoring scale. The changes were rated using scores as follows: score 0, 0% to 25% positive staining in all evaluated blood vessels; score 1, 25% to 50% positive; score 2, 50% to 75% positive; and score 3, 75% to 100% positive staining in all evaluated blood vessels.

Statistical analysis. The results were analyzed using an unpaired Student’s t tests or Mann-Whitney test. Statistical significance was defined as P < 0.05, unless otherwise stated, using SPSS.

Results

Combined treatment with F/S/G maximizes circulating bone marrow cells. To increase the number of bone marrow cells circulating in the blood, several BMC mobilizing agents and combinations were tested. G-CSF, as used previously (8), induced the circulation of CFU-GM and CAFC-day 28 cells (Fig. 1B and C). Flt-3L or SCF showed similar responses (data not shown). The combined treatment with F/S/G, however, lead to a pronounced (±10 fold) enhancement and prolonged circulation time in blood of CFU-GM (Fig. 1B) and to a somewhat lesser extent CAFC-day 28 (Fig. 1C) cells. Therefore,
F/S/G was used in further experiments for comparison with G-CSF as used before (8).

Increased engraftment of BMCs into irradiated glands after F/S/G compared with G-CSF does not lead to improved function or morphology. G-CSF, F/S/G-treated or (sham)-irradiated mice were compared to test whether the enhanced mobilization of BMCs lead to a higher number of BMCs in the gland. Sham-irradiated control glands (Fig. 2A, Normal) contained a base level of BMCs (14%; Fig. 2B). This was not changed 90 days after IR (13%; Fig. 2B), meaning that IR alone does not induce BMCs to engraft in the damaged gland. Subsequently, we compared irradiated G-CSF-treated (Fig. 2A, IR + G-CSF) or F/S/G-treated (Fig. 2A, IR + F/S/G) animals. As shown before (8), after IR + G-CSF treatment, significantly more eGFP+ cells were present in the irradiated gland 90 days after IR (49%; Fig. 2B), an effect that was further augmented after IR + F/S/G treatment (65%; Fig. 2B; \( P < 0.05 \)).

Next, to evaluate whether this enhanced number of BMCs in the submandibular gland resulted in improved tissue function and morphology, saliva production (Fig. 3A) and the number of saliva-producing acinar cells (Fig. 3B) were measured. As shown before (8), G-CSF treatment after IR significantly increased saliva production when compared with IR alone. F/S/G also ameliorated radiation-induced hyposalivation but not more than G-CSF alone. Similarly, the obliteration of periodic acid-Schiff-positive acinar cells (Fig. 3C, dark purple

\[ \text{Fig. 2. Mobilized BMCs reside in irradiated salivary glands. A, detection of eGFP}^+ \text{ BMCs in the glands of normal, irradiated untreated (IR), irradiated and G-CSF-treated (IR + G-CSF), or irradiated and F/S/G-treated (IR + F/S/G) mice 90 d post-IR. B, eGFP}^+ \text{ BMCs were present in glands of mobilized (F/S/G or G-CSF) mice in significantly higher numbers compared with nonmobilized (irradiated) mice. In the glands from the F/S/G-treated group, significantly more BMCs were present than in the G-CSF-treated group after IR. Bar, 50 \mu m. ^* P < 0.05.} \]
cells) seen 90 days after IR was significantly ameliorated after G-CSF treatment (Fig. 3B and C, IR + G-CSF). Again, F/S/G treatment also resulted in an amelioration of acinar cell loss (Fig. 3B and C, IR + F/S/G) but not significantly more than after G-CSF alone. These results show that salivary gland function and morphology were not further improved after F/S/G compared with G-CSF treatment. However, additional examination of the tissue indicated a pronounced change in blood vessel, which is further analyzed below.

**Reduction in radiation-induced vascular damage after BMC mobilization.** Radiation-induced damage to the vasculature will cause secondary damage to the tissue. Because G-CSF alone or in combination with Flt-3L or SCF has been shown to promote the formation of blood vessels after myocardial infarct (27), the vascular structure of irradiated glands was investigated. Microscopic comparison of the vasculature of glands of all groups, visualized by CD31 staining, immediately indicated differences in blood vessel morphology and were not further improved after F/S/G compared with G-CSF treatment. However, additional examination of the tissue indicated a pronounced change in blood vessel, which is further analyzed below.

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**Fig. 3.** Improved morphology and function of irradiated salivary glands after BMC mobilization. A, saliva flow rate measurements revealed significant reduction in hyposalivation in the F/S/G and G-CSF treatment group compared with untreated mice. B, quantification of the glandular acinar surface confirmed a significant protection against acinar cell loss with F/S/G or G-CSF mobilized BMCs after IR compared with untreated mice. C, gland morphology of the mice showed a pronounced loss of purple periodic acid–Schiff-positive acinar cells after IR when compared with normal (unirradiated, untreated). Duct cells are periodic acid–Schiff negative. Mobilization with either G-CSF (IR + G-CSF) or F/S/G (IR + F/S/G) prevented the loss in acinar cells. Bar, 50 μm. *, *P < 0.05.
Next, endothelial PCNA expression was determined to investigate active proliferation indicative of ongoing tissue replacement. Indeed, in irradiated glands (Fig. 5B), a significant loss in proliferating endothelial cells was observed compared with normal glands (Fig. 5B, Normal). However, this loss in PCNA-positive cells was completely abolished in G-CSF (Fig. 5B, IR + G-CSF) and F/S/G-treated glands (Fig. 5B, IR + F/S/G) where even normal numbers of cycling endothelial cells could be observed.

To evaluate whether more proliferation indeed leads to neovascularization, expression of endoglin, a transforming growth factor-β type I receptor (CD105; ref. 38), was investigated. This receptor plays a crucial role in the promotion of neovasculogenesis by inducing endothelial cells to proliferate and migrate (39, 40) and by stimulating blood cell-mediated vascular repair (41). Normal glands clearly expressed endoglin in both arterioles and capillaries (Fig. 5C, Normal). In contrast, in irradiated glands (Fig. 5C, IR), endoglin was poorly, if at all, expressed. However, a significant increase in endoglin-expressing blood vessels could be clearly observed after G-CSF (Fig. 5C, IR + G-CSF) and F/S/G (Fig. 5C, IR + F/S/G) treatment.

These results suggest that, especially after F/S/G treatment, mobilization enhances the post-IR submandibular (neo-)vascularization. Independent on the type of mobilization, the vasculature is also improved in function.

**Mechanism behind vascular repair.** Two mechanisms can be involved in the repair of vascular damage. First, BMCs could migrate to and transdifferentiate into endothelial cells and/or secrete cytokines inducing proliferation, or the administered cytokines could directly stimulate receptors on the endothelial cell surface of radiation-surviving vascular cells and stimulate proliferation.

To investigate whether neovascularization was caused by BMCs, eGFP expression in endothelium was investigated. Indeed, many eGFP+ BMCs were located at infiltration sites in irradiated glands of both G-CSF-treated and F/S/G-treated mice (Fig. 6A, yellow arrows). Several of these cells not only clearly coexpressed the endothelial cell marker CD31 (red; Fig. 6A, inset, arrowhead), but also some (~3%) of the eGFP/CD31-coexpressing cells showed capillary morphology (Fig. 6B). These were characterized as cells with a thin line of cytoplasm with a nucleus at the side surrounding an “empty” space and containing of green and red (orange). This clearly indicates the direct involvement of BMCs in the repair of vascular tissue. Further, it is known that endothelial cells express G-CSF receptors (8, 42). However, G-CSF treatment does not ameliorate vascular damage to the same high extent as F/S/G treatment. Although endothelial cells of salivary glands did not express c-Kit (data not shown), they did express Flt-3 (Fig. 6C, arrows). Therefore, next to G-CSF, also Flt-3L may directly stimulate endothelial cells to proliferate.

These data suggest that the revascularization process induced by F/S/G and to a lesser extent by G-CSF, at least partly, originates from BMCs. Coactivation pathways induced by direct activation of cytokine receptors on preexisting endothelial cells cannot be excluded. Either way, radiation-induced vascular damage can be prevented by G-CSF and especially F/S/G treatment and may contribute to the repair of IR-damaged tissues.

**Discussion**

G-CSF mobilized BMCs contribute to the repair of irradiated glands, by reducing the loss of (saliva-producing) acinar cells and improving function, but without significant differentiation
into salivary glands cells (8). This suggests that BMCs in the 
damaged gland induced radiation-surviving stem/progenitor 
cells to enhance tissue repair. In this study, we investigated 
whether a more pronounced mobilization of BMCs would 
lead to a higher number of BMCs in the irradiated gland. 
Subsequently, this would enhance the regeneration of the 
gland, resulting in an improved protection against radiation-
duced salivary gland dysfunction. We show that indeed 
enhancement of the number of circulating BMCs does yield 
more BMCs in the damaged salivary glands, but this does not 
further augment the amelioration of radiation-induced hypo-
salivation. However, we also show that vascular injury was 
reduced after F/S/G treatment by active repair even at 90 days 
after IR involving BMCs. This provides opportunities for the 
development of new strategies to potentially prevent late tissue 
damage after IR in general.

Tissue regeneration in this model is suggested to be mainly 
caused by BMC-mediated paracrine stimulation (8, 16). The 
additional BMCs induced to migrate to the salivary gland by 
F/S/G treatment does not appear to add anything to this. 
Therefore, the BMC-induced regeneration of saliva secretory 
acinar cells is probably limited by the number of stem cells 
surviving the radiation insult. Indeed, we observed3 that 
increasing the IR dose reduces the positive effect of G-CSF 
on salivary gland function.

Fig. 5. Restored expression of eNOS, PCNA, and endoglin after F/S/G/treatment in the irradiated glandular vasculature. A, eNOS is clearly expressed by endothelia of normal blood vessels and capillaries, whereas after IR only few blood vessels expressed eNOS. In the G-CSF-treated (IR + G-CSF) and F/S/G-treated (IR + F/S/G) groups, significant more eNOS-expressing blood vessels were observed. B, endothelial cells from normal blood vessels do occasionally divide as indicated by PCNA expression. After IR, hardly any PCNA expression could be detected in endothelial cells. In contrast, in glands from G-CSF-treated and F/S/G-treated mice, normal levels of dividing 
endothelial cells were found. C, normal blood vessels and capillaries are characterized by endoglin expression. Strikingly, 90 d post-IR, endoglin expression was completely 
lost. After both G-CSF (IR + G-CSF) and F/S/G (IR + F/S/G) treatments, endoglin expression reappeared. Bar, 10 mm. *, P < 0.05; **, P < 0.1.

4 Unpublished data.
Interestingly, however, the finding of repair of vascular damage after BMC mobilization may be of great importance. Late radiation-induced gland blood vessel damage (90 days post-IR) is characterized by blood vessel dilation, which contributes to decreased blood perfusion of the salivary gland (43). We show that irradiated vessels clearly lost their capacity to proliferate and showed reduced endoglin expression, which is important for neoangiogenesis (44) and a crucial factor in vascular repair (41). Furthermore, the reduced expression of eNOS in irradiated salivary glands is consistent with the lack of eNOS-mediated relaxations observed in irradiated rabbit ear arteries (45) and in cervical arteries of patients after radiotherapy (46). Interestingly, suppression of endoglin is accompanied by eNOS protein down-regulation in cultured endothelial cells (47), emphasizing the importance of our observations.

All these deleterious effects of irradiated glandular vasculature were ameliorated after BMC mobilization and most pronouncedly after F/S/G. The improved morphology not only of the large vessels but also of capillaries was due to active replacement of the endothelium. The regenerated/newly formed blood vessels, especially after F/S/G treatment, were partly bone marrow-derived, as indicated by the capillary coexpression of CD31 and eGFP. This is the first time such an observation has been reported in irradiated vessels, although it has been shown to occur after ischemia in arterioles and capillaries of the limb (48, 49) and the infarcted heart (27, 31). The more pronounced improvement of the vasculature after F/S/G treatment may be due to an enhanced mobilization of endothelial progenitor cells, which are not only capable of differentiating into endothelial cells (50) but can also release growth factors that act in a paracrine way to support the endothelium (51). Therefore, a change in composition of the mobilized BMCs may be responsible for the different effects between G-CSF alone and F/S/G. Interestingly, for potential future clinical use in humans, G-CSF alone already pronouncedly

Fig. 6. eGFP and Flt-3L receptor expression in blood vessels. A, at the infiltration site in glands of both G-CSF-treated and F/S/G-treated mice, eGFP+ BMCs (yellow arrows) were detected. Also, CD31+ eGFP+ (red, white arrows) cells were noticed. Some eGFP+ cells coexpressed CD31 (red/green, white arrowhead). B, coexpression of eGFP-expressing CD31+ capillary (arrow) was observed. C, receptor for Flt-3L in salivary glands was present on blood vessels (arrow). Bar, 20 μm (Flt-3L receptor) and 10 μm (eGFP+ CD31+).
increases circulating endothelial progenitor cells and angiogenic cells (52). An alternative or collaborative mechanism may be the direct activation of cytokine receptors inducing repair of the vasculature. Both treatment modalities included G-CSF of which receptor activation leads to migration and proliferation of endothelial cells (53). G-CSF not only mobilizes endothelial progenitor cells but also promotes angiogenesis in ischemic tissues (54, 55) through the induction of the release of angiogenic growth factors. It is unlikely that SCF directly stimulates neovascularization as, in contrast to human blood vessels (56), mouse endothelia lack the SCF receptor c-Kit (57). Further, we observed a presence of Flt-3 on endothelia of the spleen, bone marrow, and salivary gland, and Flt-3L is known to be released by endothelial cells in the bone marrow (58), suggesting that Flt-3L may also directly activate neovascularization. Therefore, combining G-CSF with SCF and Flt-3L may induce a higher number of circulating endothelial progenitor cells, which after engraftment may be stimulated directly to actively neovascularize the tissue. Apparently, a more pronounced neovascularization as induced by F/S/G treatment does not lead to an improved function of the irradiated salivary gland. In salivary glands, however, the loss of acinar tissue is the determining event for radiation-induced hyposalivation (59). Vascular damage is secondary (in time) to that and its repair may therefore not allow improvement of function when the functional units are already damaged. However, damage to the vasculature is a major factor that contributes to radiation-induced damage in several other tissues (60, 60 – 62). Our results may therefore be of relevance for tissues in which vascular damage contributes to tissue dysfunction.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Repair of Radiation-Induced Vascular Damage

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