Regeneration of irradiated salivary glands by stem cell therapy
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Cytokine Treatment Improves Parenchymal and Vascular Damage of Salivary Glands after Irradiation

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ABSTRACT

During radiotherapy for head and neck cancer, co-irradiation of salivary glands results in acute and often life-long hyposalivation. Recently, we showed that bone marrow-derived cells (BMCs) can partially facilitate post-radiation 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.

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 (Flt-3L), Stem Cell Factor (SCF), and G-CSF, termed F/S/G, as mobilizing agents. Salivary gland function and morphology was evaluated at 90 days post-irradiation by measuring the saliva flow rate, the number of acinar cells and the functionality of the vasculature.

Compared to G-CSF alone, the combined F/S/G treatment mobilized a higher number and different types of BMCs to the blood stream and gave rise to a higher number of eGFP+ cells in the irradiated submandibular gland. Both treatments reduced radiation-induced hyposalivation compared to the untreated group, with the same magnitude. Surprisingly, however, F/S/G treatment resulted in significant less damage to submandibular blood vessels and induced BMC-derived neo-vascularization.

Post-irradiation F/S/G treatment facilitates regeneration of the submandibular gland and ameliorates vascular damage. The latter is partly due to BMCs differentiating into vascular cells as well as likely direct stimulation of existing blood vessel cells by cytokines.

INTRODUCTION

Radiation-induced damage to normal tissues may result in organ dysfunction, may limit the optimal treatment dose, and/or cause a reduction in the patient’s post-treatment quality of life. Radiotherapy of head and neck cancer patients, which often involves co-irradiation of the salivary glands, induces hyposalivation with concomitant symptoms like oral dryness, dental caries, oral infections, difficulties in speech and food mastication. Although Intensity Modulated Radiation Therapy, IMRT, enables a reduction of the dose delivered to the salivary gland, many patients still suffer from salivary gland dysfunction. Therefore, a treatment to reduce post-irradiation 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 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 are typical signs of radiation-induced damage to the vasculature. Next to the reduced capillary density, dilation of larger blood vessels can induce ischemia and secondary tissue function loss.

We recently showed that treatment with Granulocyte-Colony Stimulating Factor (G-CSF), inducing mobilization of bone marrow-derived cells (BMCs) to the blood stream, resulted in the abundant presence of BMCs in irradiated salivary glands, improved morphology and reduced dysfunction. Similar observations have been made in tissues like skin, lung, kidney, liver, brain, and myocardium. However, although G-CSF treatment resulted in the improvement of salivary gland function and formation of new acinar cells, very few were bone marrow-derived. Therefore, it was suggested that the process of glandular regeneration was likely to result from BMC-mediated paracrine stimulation of radiation-surviving stem/progenitor cells, as was proposed for the infarcted heart. Mobilization protocols that induce an enhanced number and different types of circulating BMCs may augment acinar cell formation, and may also induce the circulation of BMC-derived endothelial progenitor cells, known to be involved in the promotion of angiogenesis in ischemic tissues.

Mobilization of BMCs to the blood stream can be stimulated by several hematopoietic cytokines (e.g. Flt-3 Ligand, G-CSF, SCF, TPO, IL-3), chemokines (C-X-C module, SDF-1), and antibodies against adhesion molecules (VLA-4, selectin).
Moreover, Stem Cell Factor (SCF) and Flt-3 Ligand (Flt-3L) synergize with G-CSF to stimulate the migration of hematopoietic progenitor cells 190-195. Consequently, treatment with combinations of these cytokines (G-CSF and SCF or G-CSF and Flt-3L) enhanced mobilization of BMCs 196-199, and improved regeneration of damaged organs, such as injured heart 160,162,196,199, cerebral ischemia 197, and hyperglycaemia-induced pancreas 198.

Therefore, we questioned whether an altered composition and a higher number of circulating BMCs, mobilized by a cytokine cocktail, would enhance the regenerative capacity of the gland stem/progenitor cells thereby preventing radiation-induced vascular damage, and thus resulting in an improved protection against radiation-induced salivary gland dysfunction, compared to what we previously observed with G-CSF 185.

RESULTS

COMBINED TREATMENT WITH FLT-3L/G-CSF/SCF (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 previously used 185 induced the circulation of CFU-GM and CAFC-day-28 cells (Fig. 1B and C). Flt-3 Ligand or SCF showed similar responses (not shown). The combined treatment with Flt-3L/SCF/G-CSF (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 to G-CSF as used before 185.

INCREASED ENGRAFTMENT OF BMCs INTO IRRADIATED GLANDS AFTER F/S/G COMPARED TO 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 BM-derived cells in the gland. Sham-irradiated control glands (Fig. 2A, Normal), contained a base level of BM-derived cells (14%, Fig. 2B). This was not changed 90 days after irradiation (IR) (13%, Fig. 2B), meaning that IR alone does not induce BMCs to engraft in the damaged gland. Subsequently, we compared irradiated G-CSF (Fig. 2A, IR + G-CSF) or F/S/G-treated animals (Fig. 2A, IR + F/S/G). As shown before 185, after IR + G-CSF treatment significantly more eGFP+ cells were present in the irradiated gland 90 days after irradiation (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 185, G-CSF treatment after IR significantly increased saliva production when compared to IR alone. F/S/G also ameliorated radiation-induced hyposalivation, but not more than G-CSF alone. Similarly, the obliteration of PAS+ acinar cells (Fig. 3C, dark purple cells), seen 90 days after IR, was significantly ameliorated after G-CSF treatment (Fig. 3C, IR + G-CSF). Again, F/S/G treatment also resulted in an amelioration of acinar cell loss (Fig. 3C, IR + F/S/G), but not significantly more than after G-CSF alone (Fig. 3B). Although, function and morphology were not improved after F/S/G, further examination of the tissue indicated a pronounced change in blood vessel, which is further analyzed below.

FIGURE 1. MOBILIZATION OF BONE MARROW-DERIVED CELLS. (A) A clockwise schematic representation of the experimental protocol. (B) Effect of the G-CSF and the mixture of Flt-3L, SCF and G-CSF (F/S/G) mobilization protocols on hematopoietic stem/progenitor cells as measured by the CFU-GM assay and the (C) CAFC-28 assay.
CHAPTER 3

REDUCTION IN RADIATION-INDUCED VASCULAR DAMAGE AFTER BMC MOBILIZATION

Radiation-induced damage to the vasculature will cause secondary damage to the tissue. Since G-CSF, alone or in combination with Fli-3L or SCF, has been shown to promote the formation of blood vessels after myocardial infarct, the vascular structure of irradiated glands was investigated. Microscopically comparison of the vasculature of glands of all groups, visualized by CD31 staining, immediately indicated differences in blood vessel morphology at 90 days post-irradiation. Typical abnormally dilated large blood vessels with a morphology clearly distinct from the vessels in control glands (Fig. 4A, inset, CD31+ cells are brown) were observed in the irradiated glands (Fig. 4A, IR). This effect was quantified by measuring the surface area of the blood vessels in relation to the rest of the tissue. The percentage of surface area occupied by enlarged (≥ 200 μm²) blood vessels was clearly enhanced in irradiated glands (Fig. 4B). Interestingly, after G-CSF treatment a significant improvement of surface area with dilated blood vessels was observed (Fig. 4B). This effect was significantly better after F/S/G treatment, resulting in an almost normalized vasculature (Fig. 4B). The surface area occupied by capillaries (≤ 10 μm²), as identified by their typical flattened (Fig. 4C, arrow) or circular (Fig. 4C, arrowhead) shape, was also strikingly lower in irradiated untreated glands when compared to control glands (Fig. 4D). After F/S/G treatment, however, a significantly increase in the percentage of area with capillaries (Fig. 4D) was observed, indicative of an improved vascu larization. To evaluate whether the improved morphology of the vasculature was translated into improved function of blood vessels, gland vasculature was tested for expression of nitric oxide synthase (eNOS). Through this enzyme can endothelial cells produce nitric oxide (NO), causing the surrounding smooth muscle cells to relax, resulting in vasodilation and an increased blood flow. In all blood vessels and small capillaries of the normal submandibular gland eNOS was clearly expressed (Fig. 5A, Normal). In contrast, in irradiated glands (Fig. 5B, IR) eNOS expression is very poorly, if at all, expressed. However, the endothelium of most vessels clearly expressed endoglin after G-CSF (Fig. 5C, IR + G-CSF). Again, after F/S/G treatment (Fig. 5B, IR + F/S/G) many more normal eNOS expressing blood vessels were observed in irradiated glands, indicating that also the glandular vascu lar function was improved.

Next, endothelial PCNA expression was determined to investigate active proliferation indicative of ongoing tissue replacement. Indeed, in normal glands (Fig. 5B, Normal) and irradiated glands (Fig. 5B, IR), only few, if any, endothelial cells expressed PCNA.

However, in the endothelium of most vessels PCNA was expressed after G-CSF (Fig. 5B, IR + G-CSF). Again, after F/S/G treatment (Fig. 5B, IR + F/S/G) many proliferating endothelial cells were observed as well.

To evaluate whether more proliferation indeed leads to neo-vascularization, expression of endoglin, a TGFβ type I receptor (CD105), was investigated. This endoglin plays a crucial role in the promotion of neo-vascularogenesis by inducing endothelial cells to proliferate and migrate and by stimulating blood cell-mediated vascular repair. Normal glands clearly expressed endoglin both in arterioles and capillaries (Fig. 5C, Normal). In contrast, in irradiated glands (Fig. 5B, IR) endoglin is very poorly, if at all, expressed. However, the endothelium of most vessels clearly expressed endoglin after G-CSF (Fig. 5C, IR + G-CSF). Again, after F/S/G treatment (Fig. 5B, IR + F/S/G) more endoglin expressing cells were observed in the endothelium.

These results suggest that besides a better morphology also a functional glandular (neo)vascu larization is preserved after mobilization, being most pronounced after F/S/G treatment.
MECHANISM BEHIND VASCULAR REPAIR

Two mechanisms can be involved in the repair of vascular damage. First, BMCs could travel to and trans-differentiate 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 neo-vascularization was caused by BMCs, eGFP expression of endothelium was investigated. Indeed, many eGFP+ BMCs were located at infiltration sites in irradiated glands of both G-CSF and F/S/G-treated mice (Fig. 6A, arrows). Several of these cells not only clearly co-expressed the endothelial cell marker CD31 (red) (Fig. 6A, insets), but also some of the eGFP/CD31 co-expressing cells clearly showed capillary morphology (Fig. 6B). This clearly indicates the direct involvement of BMCs in the repair of vascular tissue. It is known that endothelial cells do express G-CSF receptors 185,205. 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 re-vascularization process induced by F/S/G and to a lesser extent by G-CSF, at least partly, originates from bone marrow-derived cells. Co-activation pathways induced by direct activation of cytokine receptors on pre-existing endothelial cells can not 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 irradiation damaged tissues.

DISCUSSION

G-CSF mobilized bone marrow-derived cells (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 185. 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. Consequently, this would enhance the regeneration of the gland, resulting in an improved protection against radiation-induced 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 hyposalivation.
Interestingly, however, the finding of repair of vascular damage by G-CSF and more pronouncedly after F/S/G treatment may be of great importance. Late radiation-induced gland blood vessel damage (90 days post-irradiation) is characterized by blood vessel dilation, which contributes to decreased blood perfusion of the salivary gland 206. We show that irradiated vessels clearly lost their capacity to proliferate and showed reduced endoglin expression, which is important for neo-angiogenesis 207 and a crucial factor in vascular repair 204. 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 208, and in cervical arteries of patients after radiotherapy 209. Interestingly, suppression of endoglin is accompanied by eNOS protein downregulation in cultured endothelial cells 210, 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 of not only 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 co-expression 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 211,212 and the infarcted heart 180,198. The more pronounced improvement of the vasculature after F/S/G treatment may be due to an enhanced mobilization of endothelial progenitor cells (EPCs), which are not only capable of differentiating into endothelial cells 213, but can also release growth factors that act in a paracrine way to support the endothelium 214. 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 increases circulating EPCs and angiogenic cells 215.

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 216. G-CSF not only mobilizes endothelial progenitor cells, but also seems to promote angiogenesis in ischemic tissues 217,218 through the induction of the release of angiogenic growth factors. It is unlikely that SCF directly stimulates neo-vascularization as, in contrast to human blood vessels 219, mouse endothelia lack the SCF receptor c-Kit 220. Further, we observed presence of Flt-3 on endothelia of the spleen, bone marrow and salivary gland, and Flt-3L is known to be released by endothelium cells in the bone marrow 221, suggesting also Flt-3L may directly activate neo-vascularization.

Apparently, a more pronounced neo-vascularization 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 214. 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 a number of other tissue 183,222-224. Our results may therefore be of relevance for tissues in which vascular damage contributes to tissue dysfunction.

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**FIGURE 4. QUANTIFICATION OF RADIATION-INDUCED VASCULAR DAMAGE.** (A) CD31 expression highlighted the presence of dilated blood vessels 90 days after irradiation (IR), which are absent in normal glands (inset). (B) Quantification of the dilated blood vessel area (≥ 200 μm²) revealed a significant reduction in radiation-damaged blood vessels after G-CSF (IR + G-CSF) treatment compared to untreated irradiated (IR) glands. This effect was even more pronounced in the F/S/G group. (C) Further visualization and (D) quantification of capillaries (≤ 10 μm²) revealed a pronounced decrease in irradiated glands (IR). Glands from the G-CSF (IR + G-CSF) and F/S/G (IR + F/S/G) treated groups contained significantly more capillaries compared to untreated irradiated ones. Scale bar = 50 μm. *, P < 0.05.
FIGURE 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, while after irradiation (IR) and G-CSF-treatment (IR + G-CSF) only few blood vessels expressed eNOS. In the F/S/G-treated group (IR + FSG), normal eNOS levels were expressed. (B) Endothelial cells from normal blood vessels do occasionally divide, as indicated by PCNA expression. After irradiation (IR), no PCNA expression could be detected in endothelial cells. In contrast, in glands from G-CSF- and F/S/G-treated mice, a substantial number of dividing endothelial cells were found. (C) Normal blood vessels and capillaries are characterized by endoglin expression. Strikingly, 90 days post-irradiation (IR), endoglin expression was lost. After both G-CSF- (IR + G-CSF) and F/S/G-treatment (IR + F/S/G) endoglin expression reappeared. Scale bar = 10 μm.

FIGURE 6. eGFP AND FLK-3L RECEPTOR EXPRESSION IN BLOOD VESSELS. (A) At the infiltration site in glands of both G-CSF- and F/S/G-treated mice, eGFP+ BMCs were detected. Some eGFP+ cells co-expressed CD31 (red), while also CD31+ eGFP- cells were noticed (insets). (B) Co-expression of eGFP expressing CD31+ blood vessels was observed. (C) The receptor for Flt-3L in salivary glands was present on blood vessels (arrows). Scale bar Flk3-L receptor = 20 μm. Scale bar eGFP+CD31+ = 10 μm.
BONE MARROW TRANSPLANTATION PROTOCOL

To determine the best protocol to mobilize BMCs to the blood, normal mice were treated with 3 different cytokines or combination thereof. BMCs were mobilized by two s.c. injections of 25 μg PEG-Hu-G-CSF (Amgen Inc., Thousand Oaks, CA) given 3 days apart. The combination treatment protocol consists of PEG-Hu-G-CSF in combination with Flt-3 Ligand and SCF as described above. Untreated normal mice and normal mice with irradiated salivary glands served as controls.

Determinants of optimal progenitor mobilization protocol

To determine the best protocol to mobilize BMCs to the blood, normal mice were treated with 3 different cytokines or combination thereof. BMCs were mobilized by two s.c. injections of 25 μg PEG-Hu-G-CSF (Amgen Inc., Thousand Oaks, CA) given 3 days apart. The combination treatment protocol consists of PEG-Hu-G-CSF in combination with Flt-3 Ligand and SCF, all as described above, starting on the day of the first G-CSF injection. The combined treatment with Flt-3 Ligand, SCF, and G-CSF is depicted as F/S/G. Blood samples were taken from mice at day 3, 6, 10, and 14 after the first injection day. Hematopoietic progenitor cells were assayed as described earlier. Briefly, cells were plated in alpha-medium (STEMCell Technologies, Inc., Vancouver, British Columbia, Canada) containing 0.8% methylcellulose (Fluka, Buchs S.G., Switzerland); 30% FCS (Life Technologies, Carlsbad CA), and 10 μM 2-mercaptoethanol (Merck, Darmstadt, Germany) at concentrations varying from 104 to 5 x 104 nucleated cells/mL. Colony growth was stimulated by granulocyte macrophage colony-stimulating factor (10 ng/ml) and SCF (100 ng/ml). Cultures were plated in 35-mm polystyrene culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ), 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. This assay consists of a confluent FBMD-1 cell culture in 96-well plates (Costar, Corning, NY) overlaid with mobilized peripheral blood cells in a limiting dilution setup. Eight dilutions 2-fold apart were used with 10 replicates per dilution. The cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 20% horse serum (Life Technologies, Carlsbad CA) 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 frequencies were calculated using Poisson statistics.

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

IMMUNOHISTOCHEMISTRY

At 90 days post-irradiation of the salivary glands, animals were sacrificed for immunohistological analysis. The submandibular glands were extripated and incubated for 24 hours at 4°C in 4% buffered formaldehyde. Following dehydration, the tissue was embedded in paraffin. Five-micrometer sections were analyzed for the presence of GATA using a confocal scanning laser microscopy (Leica TCS SP2, Bannockburn IL). Counterstaining was done with 4',6-diamidino-2-phenylindole (DAPI). Acini were visualized using Periodic Acid Schiff’s base staining which detects mucins. CD31 expression was evaluated by overnight staining with anti-CD31 antibodies (BD PharMingen, San Diego, CA; 550274; 1:100) following 0.25% trypsin pre-treatment (Gibco, Invitrogen, CA; 27250-018) at 37°C, and visualization with light microscopy of diamino benzidine chromogen, using anti-rat rabbit biotine (DAKO, Carpinteria CA; E 0468) and an avidin-biotin-horseradish peroxidise complex (Vector Elite Avidin-Biotin Complex kit). To investigate the prevalence of blood vessels, labeling using an anti-Fli3 R antibody (Cell Signaling Technologies, Danvers MA; 3410), anti-CD68 (Dako, Carpinteria CA; M 0879), anti-eNOS (Santa Cruz Biotechnology, Santa Cruz CA; sc-654) following citrate or trypsin pre-treatment was performed. Co-expression of CD31 with eGFP was evaluated by labeling the tissue sections with anti-CD31 and subsequent anti-rat biotine and avidin-Cy3 (Sigma, St. Louis MO; S 6402). Nuclei were visualized using DAPI. QUANTIFICATION OF eGFP+ CELLS, ACINAR CELLS, AND BLOOD VESSELS

Tissue sections were analyzed using confocal scanning laser microscopy at a magnification of 630x. The frequency of eGFP+DAPI+ cells in 200 counted cells was enumerated. As not all salivary gland cells express eGFP in a salivary gland of untreated control C57BL/6 eGFP, the frequency of eGFP+ cells in normal transgenic mice was set at 100%. The percentage of surface area occupied by acinar cells was assessed by light microscopy (Olympus CX40, Germany) under 400x magnification using 100 squares of 0.25 mm2 each. Of each gland, five squares in three sections (top-middle-bottom) were scored, and subsequently the data were pooled.

Blood vessels were visualized using CD31 staining, and pictures of three sections of each gland were taken. Using the AnalySIS program (Olympus, Soft Imaging System, Münster, Germany), the surface area occupied by each blood vessel/capillary was measured, and blood vessels with a surface area above 200 μm2 were determined as being vasodilated blood vessels and vessels ≤ 10 μm2 as capillaries. Subsequently, the surface area of blood vessels was calculated as a percentage per measured 100 μm2 gland surface. The number of capillaries per 180,000 μm2 measured gland area was enumerated and converted into a percentage per 100 μm2.

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 using SPSS.