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Biology Contribution

Molecular Contrast-Enhanced Ultrasound Imaging of Radiation-Induced P-Selectin Expression in Healthy Mice Colon

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Summary

Using P-selectin specific and nonspecific microbubbles, this pilot study investigates the possibility of using molecular ultrasound to detect P-selectin expression as a potential imaging biomarker for radiation-induced inflammation. In a mouse model, the study results suggest that molecular ultrasound is indeed sensitive to radiation-induced P-selectin expression in the colon.

Purpose: To evaluate the feasibility of using molecular contrast-enhanced ultrasound (mCEUS) to image radiation (XRT)-induced expression of cell adhesion molecules that mediate inflammatory response to XRT in healthy mouse colon tissue.

Methods and Materials: The colons of male BALB/c mice (aged 6-8 weeks, n = 9) were irradiated with 14 Gy using a Kimtron IC-225 x-ray irradiator operating at 225 kV/13.0 mA at a dose rate of 0.985 Gy/min. The head and thorax regions were shielded during irradiation. A second control cohort of mice was left untreated (n = 6). Molecular CEUS was carried out before and 24 hours after irradiation using a VEVO2100 system and MS250 21-MHz center frequency transducer. Each imaging session comprised mCEUS imaging with P-selectin targeted microbubbles and control microbubbles targeted with an isotype control IgG. Quantification of mCEUS was carried out by measuring the differential targeted enhancement (dTE) parameter. The perfusion parameters peak enhancement and area under the curve were also extracted from the initial injection bolus. Animals were sacrificed at 24 hours and the colon was resected for immunohistochemistry analysis (P-selectin/CD31-stained vessel).

Results: For P-selectin targeted microbubble, a significant increase (40 a.u.; $P = .013$) in dTE (P-dTE) was observed in irradiated mice over 24 hours. In contrast, a nonsignificant change in P-selectin dTE was observed in control mice. For control microbubbles, no significant difference in the IgG dTE parameter was noted in treated and control animals over 24 hours. A nonsignificant increase in the peak enhancement and area under the curve perfusion parameters associated with blood volume was

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A.E.K. and K.S. contributed equally to this work.

Conflict of interest: none.

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noted in animals treated with radiation. Quantitative histology indicated significantly elevated P-selectin expression per blood vessel (36% in treated; 14% in control).
Conclusion: Our results confirm the feasibility of using mCEUS for imaging of XRT-induced expression of P-selectin as a potential approach to monitoring healthy tissue inflammatory damage during radiation therapy.

Introduction

Radiation-induced intestinal toxicities are a significant clinical concern and the main dose-limiting factor and obstacle to cure in patients receiving abdominopelvic radiation therapy (1, 2). These can result in treatment interruptions that compromise response and severely affect the quality of life of recovering patients. Hence, there is a need for tools to characterize early intestinal toxicities (3-6).

On a cellular/molecular basis, the intestinal inflammatory response involves leukocyte−endothelial cell interactions resulting in leukocyte infiltration into irradiated tissues (7). This occurs within hours after irradiation. The process involves selectins (L-, P-, E-) and immunoglobulin molecules (intercellular adhesion molecule [ICAM]-1, vascular cell adhesion molecule-1), among others. Of specific interest is P-selectin, which is expressed on the endothelial surface within seconds after irradiation (8). Early onset of P-selectin expression could serve as a molecular imaging biomarker for patients prone to gastrointestinal toxicities following radiation therapy.

Various imaging modalities are under investigation to characterize molecular biomarkers related to inflammation (5, 9). Molecular contrast-enhanced ultrasound (mCEUS) imaging has recently emerged as a promising preclinical and clinical tool to characterize expression of endothelial cell surface receptors, including those associated with inflammatory bowel disease (10, 11). In this proof-of-principle study, the feasibility of using mCEUS to detect P-selectin expression in the colon of mice after abdominopelvic irradiation is investigated.

Methods and Materials

Mouse model and treatments

This study was approved by the Stanford University Institutional Administrative Panel on Laboratory Animal Care. A total of 15 BALB/c mice (6-8 weeks old) were separated into 2 cohorts: treatment (n = 9) and control (n = 6). Treated animals received a single radiation dose of 14 Gy.

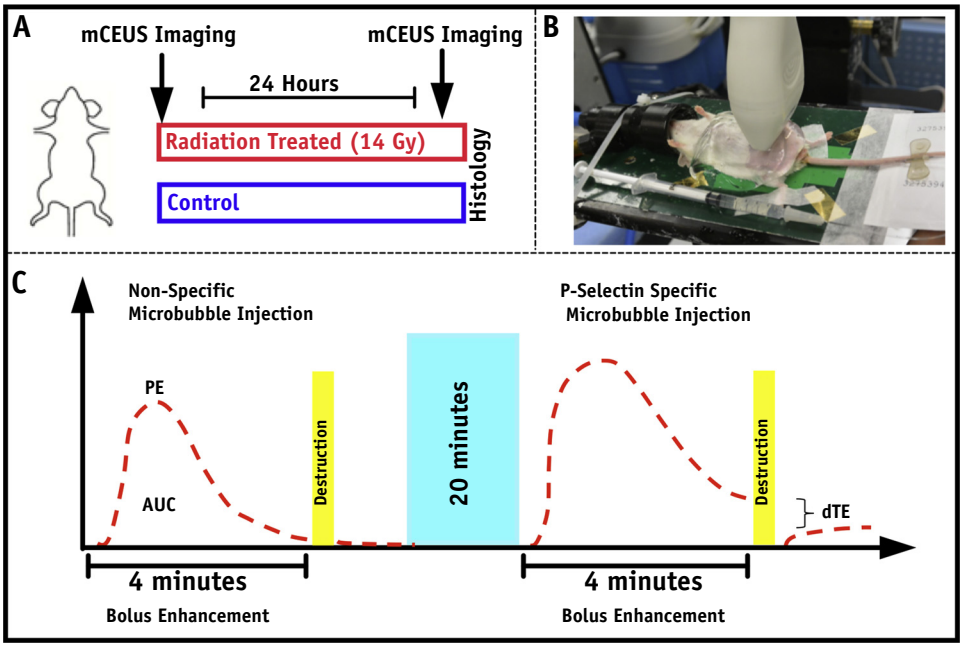

Fig. 1. (A) Treatment and imaging timeline. (B) Photograph of imaging setup, showing transducer, mouse in dorsal position, and catheter. (C) Imaging workflow for each time point, showing nonspecific and specific imaging. *Abbreviations:* AUC = area under the curve; dTE = differential targeted enhancement; mCEUS = molecular contrast-enhanced ultrasound; PE = peak enhancement.

Data acquisition

Animals were imaged with mCEUS at baseline (0 hours) and 24 hours after irradiation (Fig. 1A and B). All imaging involved specific (P-selectin targeted) and nonspecific (control) microbubbles (Fig. 1C). Imaging data were used to obtain the mCEUS dTE parameters and the bolus perfusion parameters peak enhancement (PE) and area under the curve (AUC).

Confirmatory histopathology analysis was carried out using previously established protocols to stain for the endothelial marker CD31 and associated P-selectin expression (12). For quantified histopathology, the expression of P-selectin was obtained using ImageJ (National Institutes of Health, Bethesda, MD), by determining the percentage of vessels (CD31) expressing P-selectin, as per reference 13. Detailed data acquisition methods are available in the Supplementary Text (available online at www.redjournal.org).

Statistical analysis

Quantitative parameters (dTE/PE/AUC) were evaluated for statistical significance using an unpaired t test by comparing the mean change over 24 hours (Δ) of treated to control animals (2-tailed; significant at $P<.05$ [*]). The Pearson correlation coefficient (r) and the coefficient of determination (R^2, from best-fit linear regression) between P-selectin dTE and histopathology P-selectin measurements were also calculated (n = 8). Statistical tests were conducted using Prism (version 5, GraphPad Software, La Jolla, CA).

Results

Figure 2A shows a transverse anatomic B-mode image of the colon and neighboring bladder. Figure 2B and C exhibit representative mCEUS P-selectin dTE images and

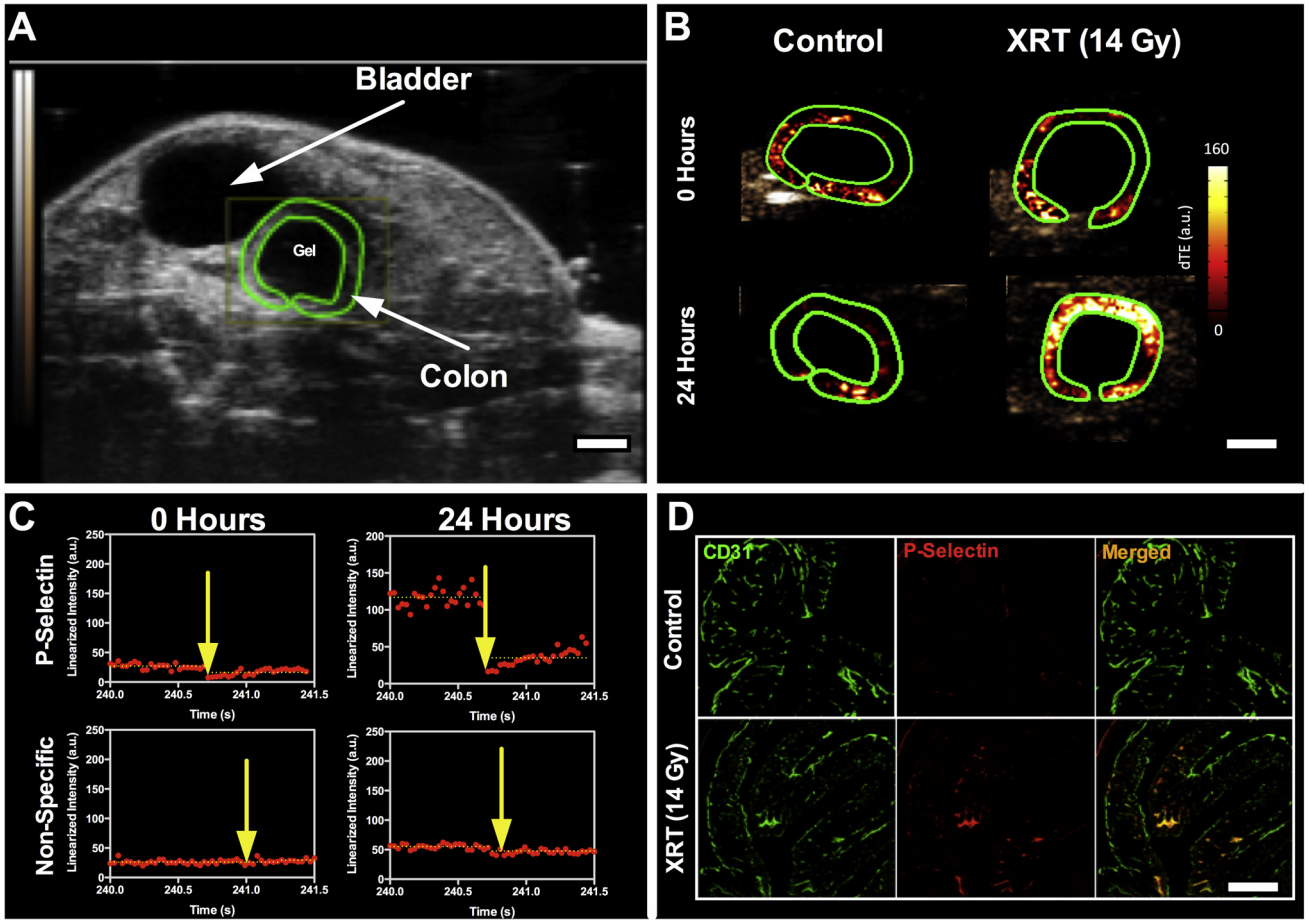

Fig. 2. (A) Representative B mode of colon region of interest and bladder. Scale bar = 1 mm. (B) Representative differential targeted enhancement maps of control and treated colon. Scale bar = 1 mm. (C) Representative TIC used to quantify differential targeted enhancement for P-selectin specific and nonspecific data. Yellow arrow is disruption pulse. (D) Representative immunohistochemistry confocal images. Scale bar = 200 μm. *Abbreviations:* TIC = Time Intensity Curve; XRT = radiation.

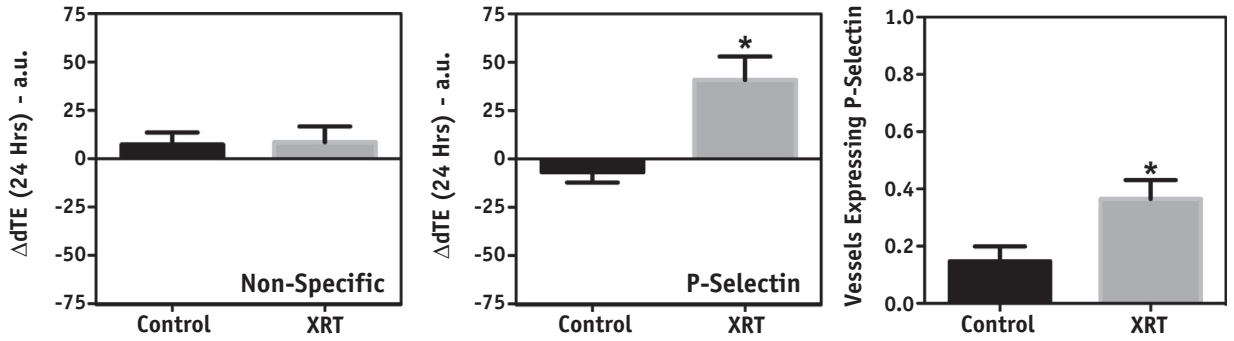

Fig. 3. Quantified ΔdTE (change over 24 hours) and immunohistochemistry with standard error of the mean. Left to right: IgG ΔdTE; P-selectin ΔdTE; percent vessels expressing P-selectin. *Abbreviations:* dTE = differential targeted enhancement; XRT = radiation. ([*] significant at $P < .05$).

quantification for 1 control and 1 treated animal, respectively. These demonstrate an increase in dTE 24 hours after irradiation. The average differences in dTE over 24 hours (ΔdTE), for control versus radiation treatment groups, are shown in Figure 3. No significant change in IgG ΔdTE is reported for treated animals (8.571 ± 8.19 a.u.) compared with control animals (7.33 ± 6.35 a.u.). A significant increase in the P-selectin ΔdTE is observed for irradiated animals (40.91 ± 12.12 a.u.; $P = .013$) compared with control animals (−6.84 ± 5.40 a.u.).

Representative images of the same CD31 and P-selectin stained colon cross-sections demonstrate a qualitative increase in the number of vessels expressing P-selectin after radiation only. In quantified histopathology (Fig. 3), minimal CD31-positive P-selectin expression is noted in control animals (0.15 ± 0.051 a.u.), whereas a significant increase in P-selectin expression is observed in radiation-treated animals (0.365 ± 0.067 a.u.; $P = .047$). Quantified histopathology resulted in a Pearson correlation coefficient of 0.724, with a significant linear correlation ($P = .021$), and an R^2 of 0.54. A nonsignificant increase in ΔPE and ΔAUC was observed in irradiated colons with both specific and nonspecific microbubbles (Fig. 4).

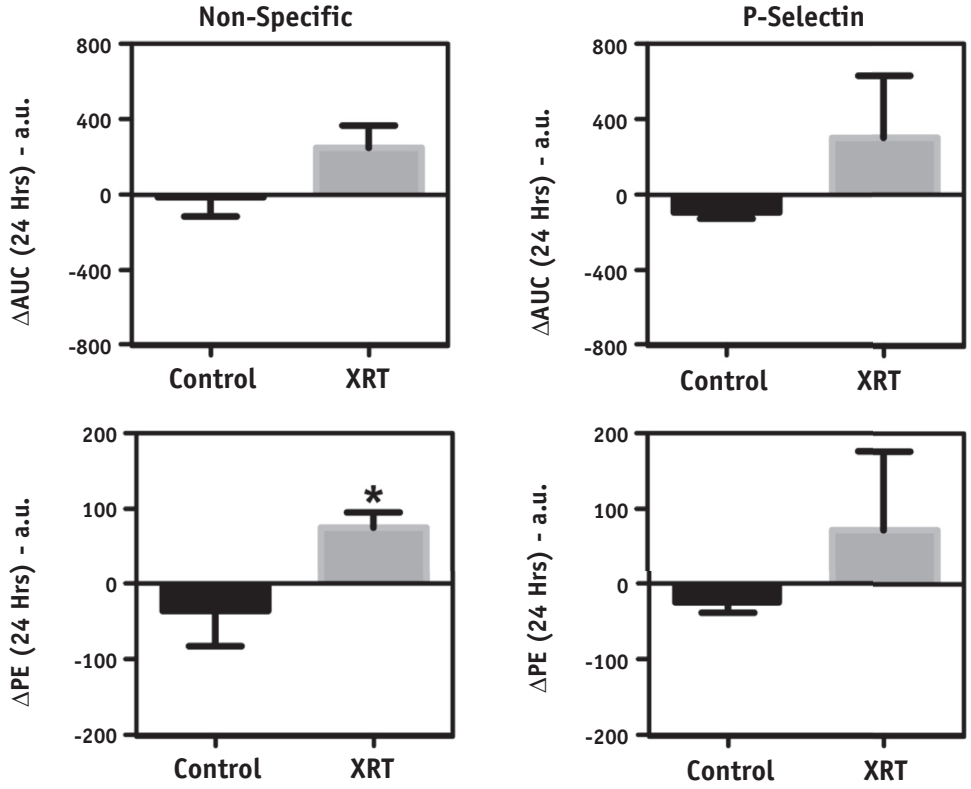

Fig. 4. Quantified bolus perfusion parameters with standard error of the mean. Top: ΔAUC for nonspecific (left) and P-selectin specific (right) conditions. Bottom: ΔPE parameter for nonspecific (IgG; left) and P-selectin specific (right) conditions. *Abbreviations:* AUC = area under the curve; PE = peak enhancement. ([*] significant at $P < .05$).

Discussion

We present the first evidence that mCEUS can detect endothelial expression of P-selectin after irradiation of healthy murine colon. A significant 24-hour P-selectin dTE increase of more than 40% was observed in treated animals. In contrast, minimal changes in P-selectin dTE were noted in untreated animals. P-selectin expression was confirmed with immunohistologic staining and correlated well with P-selectin dTE. This potentiates mCEUS for characterizing early onset of inflammation in irradiated healthy tissue, a potential clinical approach given recent introduction of translatable microbubbles targeted to P-selectin (12).

Although P-selectin's involvement in radiation-induced intestinal inflammation is known, its specific role remains uncertain (2, 8, 13). Studies have, however, confirmed that in the colon, P-selectin is critical to radiation-induced leukocyte−endothelial interactions (both adhesion and rolling) in inflammation (14, 15).

Importantly, other inflammation surface markers, such as VCAM-1 (Vascular cellular adhesion protein 1) UCAM-1 and ICAM-1 can be imaged by mCEUS (12). Indeed, a recent study has demonstrated increased endothelial binding of microbubbles conjugated to anti-ICAM-1 antibody in AT-1 prostate tumors implanted in rats after a 16-Gy carbon ion irradiation (16). Our findings thus warrant exploring mCEUS for imaging these markers in healthy tissues after irradiation.

In summary, our results suggest that endothelial P-selectin expression in the colon after irradiation can be imaged with mCEUS. Given that P-selectin expression is directly associated with colon tissue injury after irradiation, further investigations of mCEUS to identify and characterize early markers of toxicities could enable clinicians to develop new approaches to minimize radiation side effects.

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