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A Tissue Graft Model of DNA Damage Response in the Normal and Malignant Human Prostate

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Abstract

Purpose—DNA damage responses are relevant to prostate cancer initiation, progression and treatment. Few models of the normal and malignant human prostate that maintain stromal-epithelial interactions in vivo exist in which to study DNA damage responses. We evaluated the feasibility of maintaining tissue slice grafts at subcutaneous vs subrenal capsular sites in $RAG2^{-/-} \gamma C^{-/-}$ mice to study the DNA damage responses of normal and malignant glands.

Materials and Methods—We compared the take rate and histology of tissue slice grafts from fresh, precision cut surgical specimens that were maintained for 1 to 4 weeks in subcutaneous vs subrenal capsular sites. Induction of $\gamma H2AX$, p53, ATM and apoptosis was evaluated as a measure of the DNA damage response after irradiation.

Results—The take rate of subcutaneous tissue slice grafts was higher than typically reported but lower than at the subrenal capsular site. Subcutaneous tissue slice grafts frequently showed basal cell hyperplasia, squamous meta-plasia and cystic atrophy, and cancer did not survive. In contrast, normal and malignant histology was well maintained in subrenal capsular tissue slice grafts. Regardless of implantation site the induction of $\gamma H2AX$ and ATM occurred in tissue slice graft epithelium 1 hour after irradiation and decreased to basal level by 24 hours, indicating DNA damage recognition and repair. As observed previously in prostatic ex vivo models, p53 was not activated. Notably, tumor but not normal cells responded to irradiation by undergoing apoptosis.

Conclusions—To our knowledge this is the first study of DNA damage responses in a patient derived prostate tissue graft model. The subrenal capsular site of $RAG2^{-/-} \gamma C^{-/-}$ mice optimally maintains normal and malignant histology and function, permitting novel studies of DNA damage responses in a physiological context.

Keywords

prostate; prostatic neoplasms; DNA damage; H2AFX protein; human; transplants

Understanding how cells and tissues respond to DNA damage is important. Cancer arises as a result of accumulating mutations in the genome and damage to the inducible checkpoint pathway is recognized as a critical barrier against tumor formation. Furthermore, radiation therapy and DNA damage inducing drugs are widely used to treat cancer.^{1,2} DNA DSBs are considered the most harmful for genomic integrity because their unsuccessful repair leads to chromosomal aberrations. Cellular response to DSBs is characterized by dynamic translocation of DDR proteins to DNA lesions. At the DSB site histone H2A.X becomes phosphorylated (γ H2AX) by ATM kinase within seconds after the introduction of DSBs and it binds MDC1, which functions as a molecular bridge between γ H2AX and the Mre-Rad50-Nbs1 complex.³ This leads to accrual of a complex array of DDR proteins to the DSB site. The overall cellular response to DNA damage involves elegant checkpoint cascades enforcing cell cycle arrest, DNA damage repair, apoptosis and senescence.⁴

DDR research in the prostate has been limited, partly due to few realistic, representative models of the normal and malignant human prostate. We previously used primary cultures of normal HPECs in which to study DDR pathways and observed that p53 was not activated in response to DNA damage.⁵ p53, one of the most extensively studied tumor suppressor proteins, mediates cellular responses to DNA damage by activation of cell cycle checkpoints, DNA repair, apoptosis and senescence. The attenuated p53 response in HPECs suggests that the prostate lacks key DDR pathways that may be relevant to the high incidence of PCa.⁶

However, the HPEC model lacks interactions between epithelium and stroma, which are essential for PCa development and progression, and also have an important role in resistance to cancer therapy.⁷ For these reasons we developed a unique in vitro model using fresh prostate tissue slices from radical prostatectomy specimens.^{5,8} These TSCs are perhaps the most representative experimental ex vivo model of the human prostate available, containing almost all of the cells typically present in tissue and maintaining essential epithelial-stromal interactions. Using TSCs we validated the attenuated p53 response to DNA damage of HPECs in prostatic glandular epithelium of intact tissues.⁸ Furthermore, we observed differential DDR in basal vs secretory cells, emphasizing the novel information that can be gained in a multicellular tissue model.^{8,9}

While in vitro models are informative, in vivo models are considered most authentic. We recently described a method of grafting prostate tissues under the renal capsule in RAG2^{-/-} γ C^{-/-} mice.¹⁰ In this TSG model 300 mm, precision cut slices derived from primary PCa or benign tissue regain the original histology 1 month after implantation and can be maintained at least 3 months. Our goal was to evaluate the feasibility of using TSGs to study DDR after IR of benign and malignant prostate tissue, and compare grafts implanted under the subrenal capsule vs subcutaneously. Although previous studies indicated that the subcutaneous site has poor vascularization and low take rates,¹¹ this site is more easily accessible and

technically less demanding, and it has higher graft carrying capacity than the subrenal capsular site.

We describe the take rate and histology of benign and malignant human prostate TSGs at subcutaneous vs subrenal capsular sites. Furthermore, we investigated the DDR of normal, hyperplastic and tumor epithelium for the first time to our knowledge in an in vivo model of intact prostate tissue.

Materials and Methods

Patient Specimen Processing

Three histologically confirmed normal and 2 cancer tissues from radical prostatectomy specimens were used to generate TSGs at the subcutaneous site. A total of 26 normal tissue slices from 3 patients and a total of 12 tumor slices from 2 patients were grafted on the dorsal side of 19 mice with 2 slices per mouse. The 2 tumors were Gleason score 7. One Gleason score 8 cancer specimen was used to generate TSGs at the subrenal capsular site at 1 slice per mouse in a total of 4 mice.

None of the patients received prior chemical, hormonal or radiation therapy. Tissues were obtained immediately after surgery under an institutional review board approved protocol with informed patient consent. Precision cut slices 300 μ m thick and 5 mm in diameter were prepared as previously described.¹⁰

Tissue Slice Implantation

All animal studies were done in compliance with Stanford University regulations. Male 6 to 8-week-old RAG2^{-/-} γ C^{-/-} mice were bred in-house. Subcutaneous grafting was performed via a midline dorsal incision. Tissue slices were inserted beneath the dorsal skin and the incision was sealed with wound clips. Subrenal capsular engraftment was performed as previously described.¹⁰ In all mice a 25 mg testosterone pellet was inserted under the skin between the shoulder blades. The release rate of such pellets is approximately 0.2 mg per day.¹⁰

Irradiation

An x-ray irradiator was used to irradiate mice bearing TSGs at a dose rate of 126 cGy per minute. IR was directed at the subcutaneous or renal graft sites via customized jigs.

Immunohistochemistry

Grafts were recovered, fixed in 10% buffered formalin, embedded in paraffin and cut at 4 μ m. Immunohisto-chemistry was performed as previously described.⁸ The supplementary table (<http://jurology.com/>) lists the antibodies used.

Image Acquisition and Analysis

Image analysis of immunofluorescence was done as described previously.⁸ For quantification 2 to 4 fields per specimen were captured using a 40 $\times$ objective on a wide-field epifluorescence microscope. A total of 365 to 2,352 cells were analyzed per treatment for

immunofluorescence studies. Image analysis of immunohistochemical specimens was done as previously described.^{9,12} Thresholds for automated chromogen based image analysis were set such that any visually detected brown staining in the nuclei or cytoplasm was included. An average of 669 cells was quantified from 3 fields for immunohisto-chemical studies.

Results

Subcutaneous TSGs

Characterization—As an initial study, histologically normal patient tissues were engrafted subcutaneously and allowed to establish for 1, 2.5 and 4 weeks. TSGs were subsequently recovered and characterized with 1 mouse per time point and 2 TSGs per mouse. The proliferation index was determined by quantifying immunohistochemical staining for Ki67.¹² TSGs harvested at 1 and 2.5 weeks had a higher mean $\pm$ SD proliferation index than TSGs harvested at 4 weeks ($17\% \pm 9\%$ and $22\% \pm 12\%$, respectively, vs $6\% \pm 7\%$) (supplementary fig. 1, <http://jurology.com/>). Based on this kinetic study we further characterized subcutaneous TSGs 4 weeks after implantation because the proliferation index was similar to that in clinical prostate specimens.¹³

The subcutaneous site yielded a high engraftment rate in tissues from normal and cancerous prostate specimens with 87% of implants producing a viable graft (see table). This engraftment rate was significantly higher than that reported for prostate tissues implanted in SCID mice.¹⁴ Normal TSGs harvested at 4 weeks had 4 distinct morphological features, including normal glandular morphology, basal cell hyperplasia and squamous metaplasia (see table, fig. 1 and supplementary fig. 2, <http://jurology.com/>). Clinical samples served as a positive staining control for the squamous cell marker CK10 (supplementary fig. 2, <http://jurology.com/>).

In addition, 32% of TSGs had atrophic cystic structures (see table and supplementary fig. 2, <http://jurology.com/>). In cancer TSGs we identified benign glands. However, we could not document cancer based on positive staining for AMACR and negative staining for p63 despite the presence of malignant glands in TSG adjacent frozen sections, as verified by hematoxylin and eosin staining. This suggests that although the overall viability of TSGs in RAG2^{-/-} γ C^{-/-} mice was excellent, the subcutaneous site in these mice does not maintain cancer.

Normal glands and basal cell hyperplasia in subcutaneous TSGs were further characterized by immunohistochemistry. Expression of the appropriate prostate cell type specific markers was confirmed using the basal cell markers p63 and CK5, the secretory cell markers CK18 and PSA, and the stromal marker α -SMA (fig. 1). As judged by Ki67 immunoreactivity, the proliferation rate was similar and low for each glandular type and comparable to that in clinical prostate samples.¹³ Clinical prostate samples served as positive staining controls (supplementary fig. 3, <http://jurology.com/>).

DNA damage response—Four weeks after implantation mice bearing subcutaneous TSGs were sham treated (4 mice) or exposed to 4 Gy IR (10 mice) directed to the graft site with a customized jig. TSGs were harvested 1, 4, 24, 48 and 96 hours after treatment and

immunostained for the basal cell markers p63 and γ H2AX. As in our previous study,⁸ images were analyzed by Cell Image Segmentation and Classifier software, which allows for the identification of single cells and their multiplexed signal intensities.

In normal and hyperplastic glands at 1 hour γ H2AX foci were robustly induced by IR and they decreased 4 hours after IR (fig. 2). At 24 hours γ H2AX foci were largely resolved, indicating that DNA damage had been repaired (fig. 2). As measured by Ser1981 phosphorylation, the kinetics of ATM activation after IR were similar to those of γ H2AX (data not shown). γ H2AX was similarly activated in basal and secretory cells. The 2 cell types expressed equal amounts of total H2A.X (supplementary fig. 4, <http://jurology.com/>). This differs from the expression of total H2A.X in clinical prostate specimens, in which total H2A.X expression is lower in secretory cells. However, TSG luminal cells expressed classic secretory cell markers, including p27, CK18 and PSA (fig. 1 and supplementary fig. 4, <http://jurology.com/>). Staining for p53 confirmed no induction in subcutaneous TSGs after IR (data not shown), as we previously reported for prostate TSCs.⁸

Subrenal Capsular TSGs

Characterization—Four slices of Gleason score 8 PCa were implanted under the subrenal capsule of RAG2^{-/-} γ C^{-/-} mice (1 slice per mouse). TSGs were allowed to establish for a month, harvested and examined for viability, histology and cell type specific protein expression. The TSG take rate at the subrenal capsular site was 100% and all grafts were well vascularized. Subrenal capsular TSGs showed normal glands and basal cell hyperplasia. However, in contrast to subcutaneous TSGs, neither atrophic cysts nor squamous metaplasia was present. Also in contrast to subcutaneous TSGs, cancer glands were present in TSGs under the renal capsule. Immuno-staining for cell type specific markers yielded findings identical to those in our previous study.¹⁰ Briefly, basal epithelial cells expressed p63 and CK5, luminal cells were positive for CK18 and PSA, and cancer cells were negative for p63 and positive for AMACR (supplementary fig. 5, <http://jurology.com/>). As judged by Ki67 immunoreactivity, the proliferation rate was low and similar to previously published data (data not shown).¹⁰

DNA damage response—We evaluated DDR in sub-renal capsular TSGs 4 weeks after implantation. IR (4 Gy) was directed to the TSG bearing kidney in each of 2 mice and 2 control mice received sham IR. TSGs were harvested 1, 4, 48 and 96 hours after treatment and immunostained for p63 and γ H2AX. The γ H2AX response in subrenal TSGs was similar to that of subcutaneous TSGs. At 4 hours γ H2AX foci were robustly induced by IR. They decreased to the basal level at 48 hours after IR and there was no p53 induction (fig. 3, A, supplementary fig. 5, <http://jurology.com/> and data not shown). Interestingly, tumor glands had more γ H2AX foci positive cells 4 hours after IR than normal and hyperplastic glands (fig. 3, A).

Apoptotic Responses to IR

The preservation of normal and tumor glands in subrenal capsular TSGs provided an opportunity to further characterize responses to IR. We previously reported that staining for cleaved caspase-3 correlated with high and pan-nuclear γ H2AX staining in cells, which can

serve as a surrogate marker for apoptosis.⁸ Using this marker we found that tumor cells responded to IR by undergoing apoptosis at high frequency (20%) 4 hours after IR. In contrast, the frequency of apoptotic cells in IR treated normal and hyperplastic glands was low (fig. 3, *B*). The apoptotic response to IR in cancer cells was confirmed by cleaved caspase-3 staining (fig. 3, *C* and *D*). It was statistically significant in irradiated tumor tissue compared to sham treated normal glands ($p = 0.0102$).

Discussion

Our study demonstrates that thin, precision cut slices of human prostate tissue can successfully engraft in RAG2^{-/-} γ C^{-/-} mice at subcutaneous as well as subrenal capsular sites. Although we anticipated that the subcutaneous site would be suboptimal due to reports of a low take rate of minced prostate tissue grafted subcutaneously in other types of immunodeficient mice,¹¹ we predicted that using thin tissue slices in RAG2^{-/-} γ C^{-/-} mice might yield better results. Indeed, our subcutaneous grafts had an 89% take rate, better than typically reported.¹¹ However, cancer was not sustained when evaluated by hematoxylin and eosin, and p63/AMACR staining. Also, undesirable histological changes occurred in the subcutaneous TSGs, including squamous meta-plasia, basal cell hyperplasia and cystic atrophy, as described by others.¹⁵ In accord with other findings,¹⁴ the subrenal capsular grafting site had the most efficient take rate for benign and malignant tissues. The 100% take rate in our study was better than previously reported,^{14,16} perhaps because we used thin tissues, or because RAG2^{-/-} γ C^{-/-} mice are more supportive of human cells than other immunodeficient mice strains.¹⁷ We noted no squamous metaplasia or atrophic glands in sub-renal capsular grafts.

TSGs at subcutaneous and subrenal capsular sites permitted the investigation of DDR in human prostate tissue. In this more physiological context we recapitulated our earlier findings of attenuated activation of p53 in primary cultures and TSCs in response to IR. Thus, the presence of normal but nonfunctional p53 in prostate progenitor cells and tissues might explain the lack of selection for p53 gene mutations in early stage prostate tumors.^{6,18}

ATM, the major transducer of DSB signaling, regulates several hundreds of target proteins.³ As indicated by its Ser1981 autophosphorylation, ATM activity is not proliferation dependent and it is maintained even in differentiated cells.^{8,9,19–21} However, its substrate network varies depending on cell type and differentiation status. For example, we reported that H2A.X, its major target, is expressed at a much lower level in prostate secretory cells than in basal cells and this is recapitulated by their differential γ H2AX responses to IR in ex vivo cultured prostate TSCs.^{8,9} However, the IR induced γ H2AX response was similar in TSGs in each cell type, possibly reflecting similar H2A.X expression. Histone variant H2A.X isoforms are transcribed in a replication dependent and independent manner,²² indicating that expression could be influenced by cell differentiation. The chromatin epigenetic state or maturation of secretory epithelial cells in TSGs may possibly be incomplete, resulting in increased H2A.X expression despite the expression of typical differentiation markers, eg CK18, PSA and p27.

Information on the response of normal human tissue to IR in situ is limited to a few studies of skin biopsies of patients who received radiotherapy for tumors such as those of the breast and prostate.^{23–25} Coates et al engrafted breast tissue in the sub-cutaneous site of mice, enabling studies of the radiation responses of normal human breast tissue in situ.²⁶ However, most studies of DDR have been done in proliferating in vitro cell lines and the results of these experimental models may not always reflect situations in vivo. For example, Barreto-Andrade et al reported that radiosensitization by a PARP inhibitor in vitro failed to predict comparable results in tumors in vivo.²¹ Storch et al reported that 3-dimensional cell cultures and xenografts were more radioresistant than monolayer cultures.²⁷ Discrepancies among results in monolayer cultures, 3-dimensional models and xenograft studies may also be due to epithelial and stromal cell interactions that have a major role in cellular responses to different stresses.⁷ For these reasons and the facts that not only benign prostate tissues but also malignant prostate tumors have a low proliferation index and most cells are terminally differentiated and, thus, nonproliferating,¹³ there is a need to perform DDR studies in more realistic models that mimic clinical prostate tissue.

To our knowledge no one has previously studied DDR in patient derived tumor grafts. We found that IR triggered apoptosis more efficiently in tumor cells than in normal cells, which is a clinically relevant observation. Our TSG model will provide additional opportunities for novel biological and preclinical studies. For example, radiation therapy is widely used to treat localized and advanced PCa, and the combination of androgen deprivation with radiotherapy results in better long-term outcomes.²⁸ However, to our knowledge the biological basis of this clinical effect is unknown. TSGs provide a surrogate model in which to investigate the molecular and cellular mechanisms of this activity since we noted that TSGs respond to androgen deprivation in a realistic, representative manner.¹⁰ Other experimental approaches to radiosensitize PCa could also be tested in TSGs.

Furthermore, TSGs provide one of the few models in which the responses of low vs high grade tumors to DNA damaging agents can be studied. The TSG model is amenable to testing strategies for personalized medicine in the form of tailored treatment of individual patients with cancer. Complex model systems, such as those that we describe, permit better analysis of tumor heterogeneity and the heterogeneous tumor response to radiotherapy and chemotherapy. This model system may also allow for the identification and molecular characterization of therapy resistant tumor cells that lead to treatment relapse, representing a critical population to be targeted by novel therapy.

Conclusions

Prostate TSGs are maintained optimally at the subrenal capsular site in RAG2^{-/-} γ C^{-/-} mice. They provide a realistic model in which to investigate DNA damage responses in normal and cancer tissues with relevance to the initiation and treatment of prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations and Acronyms

α-SMA	α -smooth muscle actin
AMACR	α -methylacyl-coenzyme A-racemase
ATM	ataxia telangiectasia mutated
CK	cytokeratin
DDR	DNA damage response
DSB	double strand break
HPEC	human prostatic epithelial cell
IR	ionizing radiation
PCa	prostate cancer
PSA	prostate specific antigen
TSC	tissue slice culture
TSG	tissue slice graft

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Figure 1.

Cell type specific marker staining of normal and hyperplastic areas in subcutaneous TSGs harvested 4 weeks after implantation. Prostate cell type specific markers p63/ AMACR, CK5, CK18, PSA, Ki67 and α -SMA were detected by immunohistochemistry. Reduced from $\times 10$, scale bar indicates 100 μ m. Insets, fivefold enlargement.

Figure 2.

DNA damage response of human prostate tissue to IR in subcutaneous TSG of RAG2^{-/-} γ C^{-/-} mouse host. Subcutaneous grafts were sham treated or irradiated with 4 Gy 4 weeks after implantation. Tissues were harvested after treatment, fixed and stained for p63 and γ H2AX. Nuclei were counterstained with Hoechst 33342 for DNA. Confocal images show normal (A) and hyperplastic (B) areas of representative fields. *Mock*, sham treated. Reduced from $\times 63$, scale bars indicate 50 μ m. Insets, 3.8-fold enlargement. Quantitative image analysis of γ H2AX foci positive cells acquired from 2 to 4 fields per specimen and quantified using imaging application (C). Total of 663 to 2,430 cells were analyzed per time point and gland type. *h*, hours. Error bars indicate SD.

Figure 3.

DNA damage response of human prostate tissue to IR at subrenal capsular graft site of RAG2^{-/-} γ C^{-/-} mice. TSGs implanted under renal capsule were sham treated (*Mock*) or irradiated with 4 Gy 4 weeks after implantation. Tissues were harvested after treatment, fixed and stained for p63 and γ H2AX. Nuclei were counterstained with Hoechst 33342 for DNA. Quantitative image analysis of DNA damage by γ H2AX staining (A), and apoptosis of normal, hyperplastic and tumor areas (B). Images were acquired from 2 to 4 fields per specimen and quantified by imaging software. Total of 365 to 1,371 cells were analyzed per time point and gland type. *h*, hours. Error bars indicate SD. Double asterisks indicate Student 2-tailed t-test with 2-sample unequal variance $p < 0.01$ vs control. Triple asterisks indicate Student 2-tailed t-test with 2-sample unequal variance $p < 0.001$ vs control. Representative immunohistochemical staining for cleaved caspase-3 of normal, hyperplastic and tumor areas of TSGs sham treated or irradiated with 4 Gy 4 weeks after implantation (C). Tissues were harvested after treatment, fixed and stained for cleaved caspase-3. Reduced from $\times 40$, scale bar indicates 50 μ m. Representative quantitative image analysis of cleaved caspase-3 in 3 fields per specimen quantified by FrIDA (D). Error bars indicate SD. Single asterisk indicates Student 2-tailed t-test with 2-sample unequal variance $p < 0.05$ vs control.

Overall recovery rate of grafted human prostate tissue at subcutaneous site in RAG2^{-/-}γC^{-/-} mice

	Normal Graft	Tumor
No. implanted	26	12
No. recovered	23	10
% Take	89	83
% Cystic	31	33
% Basal cell hyperplasia	65	58
% Squamous metaplasia	62	25