ANTICANCER RESEARCH *43*: 335-341 (2023)
doi:10.21873/anticanres.16168

Use of Conditioned Extracellular Matrix as a Tissue-engineered Tumor Matrisome for Prostate Cancer and Melanoma Immunotherapy

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Abstract. *Background/Aim: Decellularized extracellular matrix (ECM) acts as a depot for biochemical factors when conditioned by the growth of cells that are subsequently removed, and in the case of tumors, this ECM depot is known as the matrisome. This study was undertaken to determine whether a tissue-engineered matrisome could be used as an antigenic depot to stimulate protective immunity against tumor regrowth and metastasis following surgical reduction of the tumor. Materials and Methods: Using two transplanted tumor cell models, the PAIII rat model of prostate cancer and the B16F1 mouse model of melanoma, mice were administered either media (control), a suspension of inactivated tumor cells, extracellular matrix (SIS), or a matrisome engineered through growth and removal of tumor cells on SIS that was then implanted either directly onto the resected tumor bed or at an anatomical site distant to the tumor bed. Tumor weights were determined at 21 days (rats) and at 17 days (mice), and the number of metastatic foci on the lungs were enumerated at 21 days in rats. Results: Data showed that for both PAIII and B16F1 tumors, mean PAIII and B16F1 tumor weights were significantly reduced for vaccinated animals compared to controls. Furthermore, significantly fewer metastatic foci from PAIII tumors were present on the lungs in vaccinated rats compared to controls. Conclusion: Antigens within the tissue-engineered matrisome stimulated an inhibitory response to tumor growth; this strategy should be explored further as a means of cancer immunotherapy.*

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Key Words: Immunotherapy, cancer, tissue vaccine, matrisome, Lobund-Wistar rat, melanoma, prostate cancer.

Extracellular matrix (ECM) is an essential component of all tissues and provides strength, as well as serving as a lattice for cell attachment and growth. Though long regarded as a passive structural feature of tissues, ECM has more recently been understood to play a dynamic, interactive role with the cellular component of tissues. Composed largely of the structural family of collagen proteins, ECM has been demonstrated to be rich in a variety of other bioactive molecules, such as hyaluronan, heparan sulfate, chondroitin sulfate A, dermatan sulfate, fibroblast growth factor-2, and vascular endothelial growth factor (1-3). In this way, then, ECM both receives and sends biomechanical and biochemical cues to the cells that are part of its environment, and this complex meshwork of macromolecules collectively comprises and defines the matrisome.

ECM plays a critical role in cancer growth and progression. Tumor cells modify their microenvironment by elaborating factors that favor ECM remodeling in a way that supports tumor growth. For example, increased stiffness of ECM is associated with tumor growth and spread (4, 5). In this regard, it is known that progression of a variety of tumors, including cancers of the breast and pancreas, is associated with tumor cell over-expression of lysyl oxidases – enzymes responsible for collagen cross-linking in the ECM (6, 7). Along with other matricellular proteins, such as osteonectin, tenascins, periostin, hyaluronan, versican, and bioactive ECM fragments known as matrikines, the tumor matrisome embodies a rich depot of biomolecules that represent potential targets for therapeutic intervention.

Building upon the idea that the immune system might be effectively harnessed to combat cancer, recent strategies have examined the potential clinical utility of immunotherapy. Approaches have included the use monoclonal antibodies directed against specific tumor antigens or employed as immune checkpoint inhibitors, *ex vivo* stimulation of autologous T lymphocytes or dendritic cells with transfer back to the host, CAR-T cell therapy, and immunization with inactivated allogeneic cancer cells (8-14). While some studies have shown encouraging results, such approaches are generally limited by the presentation of a relatively small number of

antigens to the immune system. Because tumors represent complex tissues, approaches that offer a broader menu of antigenic targets to the immune system might offer the possibility of broader attack vectors for therapeutic success.

Tissue vaccines produced directly from harvested tumor tissue offer an enormous repertoire of antigens, including those associated with the matrisome. In preclinical models, tissue vaccines that are produced in a way to include both killed, autologous tumor cells, and matrisome have demonstrated a strong safety profile and inhibition of prostate tumor growth and metastasis (15-20). However, a significant challenge with tissue vaccines is the limited amount of harvested tumor material that is sometimes available for processing into a vaccine.

Therefore, we decided to take a tissue engineering approach to creating a larger quantity of tumor-specific matrisome using culturing and then decellularization processing techniques. We describe herein experiments undertaken to create a synthesized, tissue-engineered tumor matrisome therapeutic as the means to capture a broad range of antigens that might be used to direct an antitumor response, which does not depend on a finite amount of harvested tumor tissue, and has the potential to be shelf-stable for future medical uses.

Materials and Methods

Animals. Studies were conducted using both rats and mice. Ten- to twelve-week-old male Lobund Wistar (LW) rats were obtained from a breeding colony maintained at the University of Notre Dame. The LW rat is an established model of prostate cancer that metastasizes to the lungs, and from which a transplantable prostate cancer cell line (PAIII cells) has been established (21, 22). Female C57BL6 mice of 6-8 weeks of age (Envigo, Indianapolis, IN, USA) were used for studies involving mice. All animals were serologically free of common rodent pathogens. Animals were housed on hardwood bedding in individually-ventilated caging systems (Allentown, Inc., Allentown, NJ, USA) in rooms with a 12:12 light:dark cycle. Both rats and mice were provided *ad libitum* access to reverse osmosis water and Teklad 7001 4% Fat Mouse/Rat Diet (Envigo). All animal studies were approved by the Institutional Animal Care and Use Committee.

Tumor growth. Tumors were grown in both rats and mice for production of the vaccines and for creation of tumor-bearing animals to be used in vaccine evaluation. In this regard, two tumor models were used: the PAIII prostate tumor of LW rats; and the B16F1 mouse melanoma.

PAIII cells were originally isolated from an autochthonous, metastatic prostate adenocarcinoma in a LW rat (23). The cells were maintained as tumors by serial passage of tumor samples in LW rats. Passage of tumors was performed by harvesting a 3-g portion of tumor from a euthanized rat and mechanically dissociating the tumor material through an 80-mesh screen in 10 ml of Modified Eagle's Medium (MEM). Subcutaneous administration of 300 μl of this cell suspension to syngeneic rats consistently resulted in tumor masses, which could be palpated as early as 7 days after cell suspension administration, and which typically become large subcutaneous tumors weighing in excess of 10 g that metastasize to the lungs within 40 days (21). This same cell suspension was used for seeding ECM with PAIII cells in culture.

The B16F1 mouse melanoma model consistently results in tumors that are not metastatic when tumor cells are administered subcutaneously (24, 25). Following an established procedure, cells were grown to confluence in DMEM media at 37˚C in a CO_2 incubator (26). Following trypsinization, cells were suspended in DMEM, and 1×10^5 tumor cells in 100 μl of DMEM were administered subcutaneously to the flanks of mice. Palpable tumors were present within 7 days in all mice. Mice were euthanized and tumors harvested 14 days after administration of tumor cells, and the tumors were dissociated through an 80-mesh screen into 10 ml of DMEM. The resulting cell suspension was used to seed ECM for culture preparation of melanoma matrisome samples; and for production of melanoma tumors for testing of matrisome anti-tumor activity. Vaccines were produced by subcutaneous administration of 100 μl of this suspension to syngeneic mice.

Preparation of vaccines. To evaluate the potential induction of anti-tumor immunity by the tumor matrisome, two conditioned ECM preparations were made, one for LW rat prostate cancer and one for B16F1 mouse melanoma. Cell-conditioning of ECM is well described (27, 28) and involves the tissue-engineered approach of culturing a cell population on an ECM, allowing these cells to deposit new, cell-specific matrisome components, and using a minimally-disruptive decellularization process to strip the cells away and leave the newly formed matrisome intact.

Prostate cancer matrisome and melanoma matrisome were produced by incubating 3×10^6 PAIII LW rat prostate cancer cells or 1×10^6 B16F1 mouse melanoma cells (American Type Culture Collection, Manassas, VA, USA), respectively, on 2 cm × 2 cm sections of SIS (medical-grade, acellular submucosa of porcine origin) in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum for 72 h at 37˚C in a CO_2 incubator. Small intestinal submucosa (SIS; Biodesign® Soft Tissue Graft, Cook Biotech Inc., West Lafayette, IN, USA) was provided as a sterile, lyophilized sheet of ECM, and preconditioned with serum-containing culture media for 24 h prior to seeding.

Following incubation, cells were removed from the newly formed matrisome by placement of the entire construct in 20 ml of 1.0 M KSCN for 24 h at 37˚C, followed by extensive washing with Tris buffer. To confirm the presence of cells before lysing and absence afterward, samples of the matrisome were fixed in 10% formalin, embedded in paraffin, sectioned at 4-5 μm thickness, stained with hematoxylin and eosin, and examined microscopically.

As positive controls, glutaraldehyde-fixed tissue (GFT) vaccines were prepared as previously described (16). Briefly, a prostate GFT vaccine preparation was prepared by harvesting from a Lobund–Wistar (LW) rat, 3 g of a subcutaneous tumor produced by administration of prostate adenocarcinoma cells, which were originally isolated from a *de novo*, metastatic prostate adenocarcinoma in a LW rat (23). The tissue was finely minced, and the cells separated using an 80-mesh screen to create a cell suspension in MEM. The cell suspension was incubated in 2.5% glutaraldehyde (v/v) at 37˚C for 60 min and then washed thoroughly with medium to produce the vaccine. Mouse B16F1 melanoma GFT was produced by analogous processing of harvested mouse melanoma tumors.

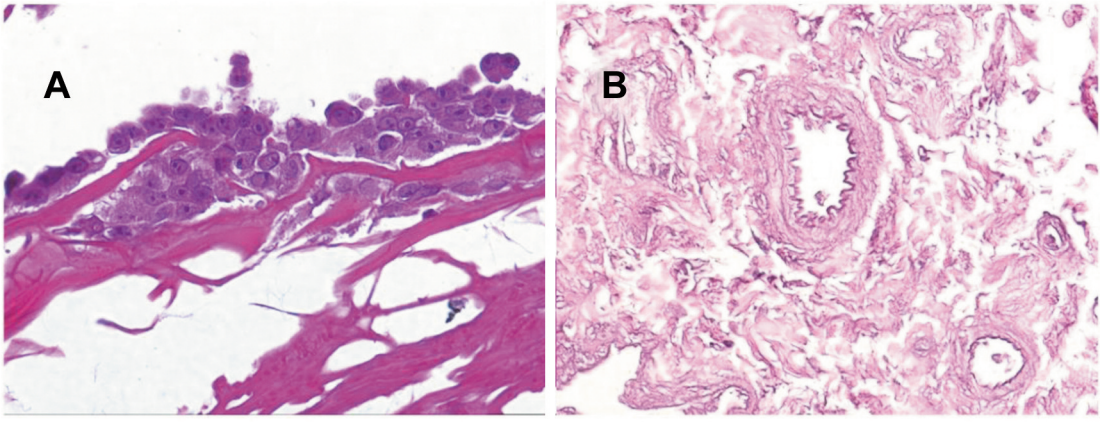

Figure 1. *Micrograph of tissue showing PAIII tumor cells grown on the small intestinal submucosa (SIS), an extracellular matrix material derived from porcine small intestinal submucosa. Nuclei of cells populating the SIS (A) are evident and in contrast to acellular SIS following removal by potassium thiocyanate treatment (B).*

Study design. The study was designed to test the hypothesis that the tissue-engineered tumor matrisome could prevent regrowth of tumors following surgical tumor excision. Forty PAIII tumor-bearing rats at day 14 after tumor cell administration and 40 B16F1 tumor-bearing mice at day 18 after tumor cell administration were anesthetized with inhaled isoflurane administered *via* a precision vaporizer. Following preparation for aseptic surgery, subcutaneous tumors were excised to visually normal tissue margins. Prior to closure of the surgical wound, animals were divided into groups of 10 each that were treated in a blinded manner with either 0.25 ml of DMEM (negative control); GFT vaccine (1×10^6 fixed PAIII or B16F1 tumor cells in 0.25 ml of DMEM); a PAIII matrisome (rats) or a B16F1 matrisome (mice) applied directly to the tumor bed; or a PAIII matrisome (rats) or a B16F1 matrisome (mice) applied subcutaneously in the dorsal thorax, distant to the tumor bed. Animals were dosed with sustained release buprenorphine (Buprenex®, Reckitt Benckiser Pharmaceuticals, Slough, UK) for postoperative analgesia and closely monitored to ensure normal postoperative recovery. Using this aggressive tumor excision model, we previously demonstrated that tumor growth could be inhibited by immunization with a section of SIS on which PAIII cells were grown and fixed in place using glutaraldehyde (29).

Animals were euthanized by CO_2 overdose at 21 days (rats) and 17 days (mice) following the tumor resection and test article implantation surgery. Tumors were excised to their visibly obvious margins and weighed, and the PAIII metastatic foci on the pleural surfaces of the lungs enumerated by a prosector blinded with respect to treatment group. Group differences in mean tumor weights and the number of metastatic foci on the pleural surfaces of the lungs were evaluated for significance using the Bonferroni multiple comparisons test with significance reached when $p\leq0.05$.

Results

Histological examination of the matrisome. Samples of the culturing matrisome were examined to establish that cells had attached to ECM and were subsequently removed, resulting in an acellular matrisome. Microscopic examination showed growth of cells on SIS and an absence of cells after treatment with KSCN, thus confirming a close interaction of the tumor cells with the SIS. Cell nuclei were present in both the collagen substance and the walls of remnant blood vessels, indicating that cells from the tumor tissue had readily colonized SIS (Figure 1A). In contrast, microscopic examination of samples following treatment with KSCN demonstrated an absence of cell nuclei, indicating decellularization of the matrisome (Figure 1B).

Inhibition of tumor growth by the tissue-engineered tumor matrisome. To evaluate the ability of the tissue-engineered matrisome to inhibit tumor growth, subcutaneous PAIII prostate tumors from LW rats and B16F1 melanoma tumors from C56BL6 mice were surgically resected and the tumor bed directly treated with either media, GFT vaccine, SIS, or the tissue-engineered matrisome. Previous studies have established that treatment with GFT vaccine reduces tumor regrowth following surgical debulking in the LW rat prostate cancer model (16, 29). As shown in Figure 2, treatment of the rat PAIII tumor bed with either GFT vaccine or PAIII tumor matrisome placed directly on the resected tumor bed or subcutaneously distant to the tumor bed (14.9±0.7 g, 13.5±0.8 g, and 13.6±0.5 g, respectively) significantly ($p\leq0.01$) reduced the mean weight of recurrent tumors compared to media-treated (26.7±0.7 g) and compared ($p\leq0.05$) to SIS-treated controls (20.7±0.5 g), with no significant difference in tumor weight between the GFT- or matrisome-treated groups. The SIS-treated group had a mean tumor weight that was significantly ($p\leq0.05$) less than that for the media-treated controls and significantly more than that for the other groups. Similar results were demonstrated in the B16F1 mouse melanoma model in which mean tumor weights for the melanoma GFT vaccine (2.1±0.2 g), the B16F1 melanoma matrisome applied directly to the tumor bed (1.8±0.2 g), and

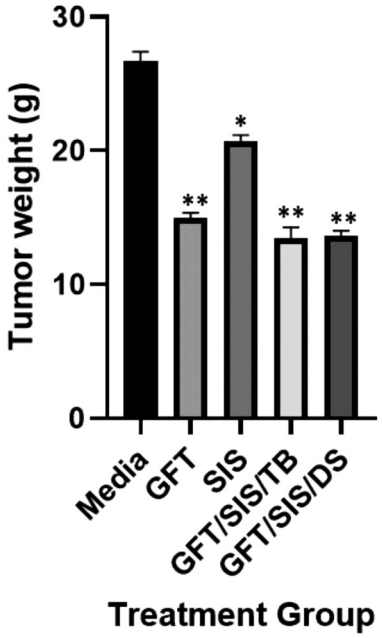

Figure 2. *Reduction in regrowth of subcutaneous PAIII tumors following surgical resection. Compared to media-treated controls (Media), mean weights of tumors regrown following surgical resection were significantly less for rats treated with SIS placed directly onto the tumor bed (SIS); a tumor-derived tissue vaccine (GFT); PAIII tumor matrisome placed directly on the resected tumor bed (GFT/SIS/TB); or matrisome placed subcutaneously distant to the tumor bed (GFT/SIS/DS). (*p≤0.05; **p≤0.01). The latter three groups had mean tumor weights that were significantly (p≤0.05) smaller than those for the SIS-treated group. Treatment group abbreviations are SIS (acellular small intestinal submucosa); glutaraldehyde-fixed tumor tissue (GFT); SIS on which tumor tissue was grown and then removed prior to placement on the resected tumor bed (GFT/SIS/TB); and SIS on which tumor tissue was grown and then removed prior to placement at a site distant to the tumor (GFT/SIS/DS).*

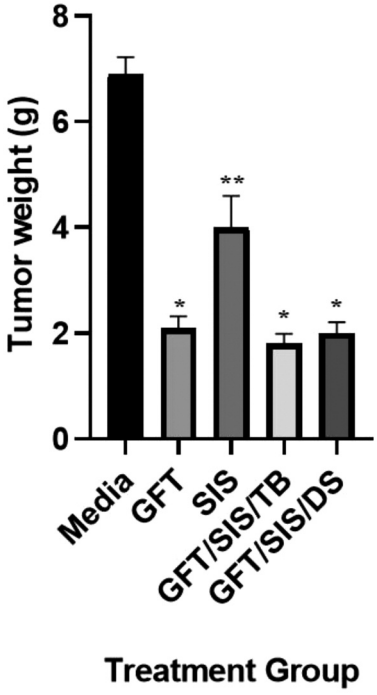

Figure 3. *Reduction in regrowth of subcutaneous B16F1 melanoma tumors following surgical resection. Compared to media-treated controls (Media), mean weights of tumors regrown following surgical resection. Mean tumor weights for the melanoma GFT vaccine (GFT), the B16F1 melanoma matrisome applied directly to the tumor bed (GFT/SIS/TB), and the B16F1 melanoma matrisome applied distant to the tumor bed (GFT/SIS/DS) were significantly less compared to media-treated controls and to animals treated with SIS. In addition, placement of SIS directly onto the resected tumor bed resulted in melanoma tumor weights significantly (p≤0.05) less than media treated controls, but greater (p≤0.02) than those treated with GFT, GFT/SIS/TB, or GFT/SIS/DS. The latter three groups had mean tumor weights that were significantly (p≤0.05) smaller than those for the SIS-treated group. Treatment group abbreviations are SIS (acellular small intestinal submucosa); glutaraldehyde-fixed tumor tissue (GFT); SIS on which tumor tissue was grown and then removed prior to placement on the resected tumor bed (GFT/SIS/TB); and SIS on which tumor tissue was grown and then removed prior to placement at a site distant to the tumor (GFT/SIS/DS).*

the B16F1 melanoma matrisome applied distant to the tumor bed (2.0±0.2 g) were significantly ($p \leq 0.01$) less compared to media-treated controls and to animals treated with SIS ($p \leq 0.2$) (Figure 3). Once again, placement of SIS directly onto the resected tumor bed resulted in melanoma tumor weights significantly ($p \leq 0.05$) less than media treated controls and greater than the other groups.

Inhibition of metastasis by the tissue-engineered tumor matrisome. To assess the ability of tissue-engineered matrisome implanted onto the tumor bed of a surgically resected tumor to inhibit metastasis, the number of metastatic PAIII tumor foci were counted on the pleural surfaces of all lung lobes in rats. The mouse melanoma B16F1 model does not typically metastasize from primary subcutaneous tumors.

As shown in Figure 4, rats that had media placed upon the resected tumor bed had a mean of 33 foci on the pulmonary surface (±4.50 SD). In contrast, rats treated with SIS only had a significant ($p \leq 0.05$) reduction in metastatic foci number (mean 20.3±2.21 SD) compared to the media control; and significantly ($p \leq 0.001$) fewer numbers of metastatic foci were noted compared to media controls in rats treated with GFT vaccine (mean 6.80, SD ±1.55), matrisome placed directly on the resected tumor bed (mean 8.30, SD ±2.83), or matrisome implanted distant to the resected tumor bed (mean 7.50, SD ±2.64). Treatment with SIS only resulted in significantly ($p \leq 0.05$) more metastatic foci compared to treatment with GFT vaccine or matrisome placed either directly on, or distant to, the resected tumor bed.

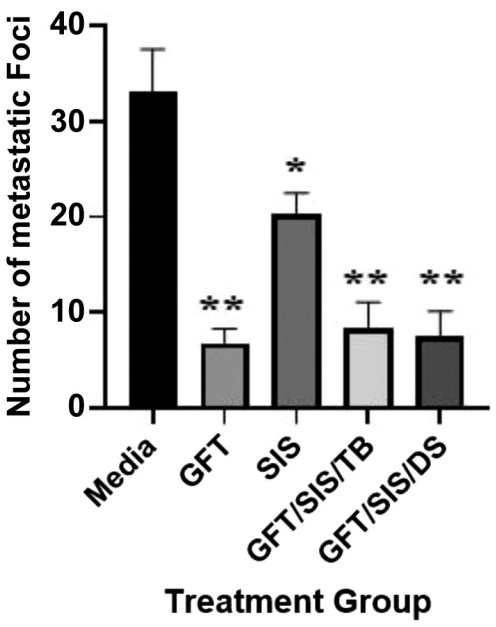

Figure 4. *Reduction in the number of PAIII metastatic foci on the surface of the lungs. Compared to control rats that were treated with media placed directly onto the resected tumor bed (mean of 33 foci, ±4.50 SD), rats treated with the decellularized extracellular matrix (SIS) only had a significant (p≤0.05) reduction in metastatic foci number (mean 20.3±2.21 SD); and significantly (p≤0.001) fewer numbers of metastatic foci were noted compared to media controls in rats treated with a tumor-derived tissue vaccine (GFT) (mean 6.80, SD ±1.55), matrisome placed directly on the resected tumor bed (GFT/SIS/TB; mean 8.30, SD ±2.83), or matrisome implanted distant to the resected tumor bed (GFT/SIS/DS; mean 7.50, SD ±2.64). Treatment with SIS only resulted in significantly (p≤0.05) more metastatic foci compared to treatment with GFT vaccine or matrisome placed either directly on, or distant to, the resected tumor bed. Treatment group abbreviations are SIS (acellular small intestinal submucosa); glutaraldehyde-fixed tumor tissue (GFT); SIS on which tumor tissue was grown and then removed prior to placement on the resected tumor bed (GFT/SIS/TB); and SIS on which tumor tissue was grown and then removed prior to placement at a site distant to the tumor (GFT/SIS/DS).*

Discussion

Immunotherapy offers an approach to cancer treatment that specifically harnesses the patient's own defenses, thus avoiding the adverse events and diminished quality of life associated with chemotherapy or radiation therapy. Both passive and active immunization approaches have been attempted with varying degrees of success. Monoclonal antibodies that target tumor cell evasion of immune checkpoints or which target specific tumor cell antigens have been used effectively to treat a variety of cancers (30). For example, anti-CD20 monoclonal antibody has contributed to remarkable success in the treatment of chronic lymphocytic leukemia, and results are improved when combined with monoclonal antibody to Bruton tyrosine kinase, a protein that plays a key role in B lymphocyte development (31). Active immunization with specific antigens alone or in combination with chemotherapy has been used to stimulate anti-tumor immunity to some cancers. For example, a cocktail of seven peptides identified by cDNA microarray profiling prolonged survival in some patients when combined with tegafur-uracil plus leucovorin (32). Likewise, administration of dendritic cells pulsed with a tumor-associated antigen (WT1) was shown to stimulate cytotoxic T-lymphocytes in patients with pancreatic cancer (10).

In contrast, tissue vaccines recognize that tumors vary greatly between individuals and that the best antigenic repertoire for an immune response is one that includes an array of antigens specific to that individual's tumor. Because tissue vaccines are produced directly from harvested tumor tissue, they include antigens associated with a diverse population of tumor cells and the supporting ECM matrisome. Inclusion of autologous tissue in a preparation designed to elicit an immune response carries the concern of auto-immunity; however, clinical studies in dogs, cats, and horses have demonstrated SIS-adjuvanted tissue vaccines to have a strong safety profile (15, 33, 34).

The interaction between ECM and associated cells is dynamic, with each influencing the other. Badylak *et al*. demonstrated SIS to be capable of supporting *in vitro* growth for a variety of cell lines, including a rat osteosarcoma line (35). Coculturing of mouse fibroblasts with keratinocytes demonstrated distinctive spatial orientation between the two cell types, providing evidence of the ability of decellularized SIS to support and expand a mixed cell population. While the ECM modulates the behavior of cells through biomechanical cues, it also serves as a depot of biochemical cues; and, in this regard, possibly also as a depot of antigens deposited by growing cells.

Woods *et al*. showed that human umbilical vein endothelial cells (HUVEC) grew more robustly when reseeded on SIS that had earlier undergone HUVEC culture and removal than on naïve SIS and was characterized by enhanced organization of cell junctions and increased metabolic activity, suggesting that growth of cells on SIS modified the material in ways that had significant biological effects (27). This same study demonstrated reduced platelet adhesion to cell-conditioned SIS, suggesting that conditioning SIS with cell-secreted basement membrane proteins could reduce thrombogenic potential. The ability of SIS to absorb, retain, and protect from degradation a variety of bioactive molecules establishes the possibility of a depot role for SIS *in vivo* (36, 37). In the same way, we hypothesized that growth of tumor cells on SIS introduces, into the ECM, antigens that are capable of stimulating anti-tumor immunity once the cells have been removed, and that this tissue-engineered tumor matrisome can be used as a therapeutic device when combined with surgical tumor debulking.

No attempt was made to determine what new matrisome components were added to the SIS by the cells in culture. The complex nature of the SIS itself as well as the very likely complexity of the deposition products made such an endeavor outside the scope of this study. Nonetheless, the determination of the components, ratios, and configurations of the newly formed matrisome represents a new frontier in understanding cell-matrix interactions both for *in vivo* therapies and for *in vitro* research or process development.

The tumor resection model provides a system that mimics the tumor microenvironment likely to occur in the clinical situation in which complete removal of the primary tumor is unsuccessful. In such cases, regrowth of residual tumors and metastatic disease often become especially aggressive (38). Non-conditioned SIS has also been shown to reduce the regrowth of PAIII tumors following resection (39), and our results confirmed that finding; however, treatment with the tissue-engineered matrisome significantly reduced tumor regrowth beyond that achieved by SIS alone.

Using models of two important cancers, we have demonstrated similar reductions in tumor growth, suggesting induction of anti-tumor immunity. That the antitumor effect was equivalent to that achieved using the GFT vaccine, further supports the idea that induction of anti-tumor immunity by the tissue-engineered matrisome is an effective adjunctive modality to surgical tumor excision. The potential benefit of the matrisome therapeutic is that more vaccine specific to the individual can be "manufactured" and thus used for more extensive or longer lasting treatment regimens. Though further studies are needed to more fully characterize and optimize the anti-tumor immune response that is induced by the tissue-engineered matrisome, the studies described here establish the possibility that this method represents a novel approach to the treatment of cancer using distinctly different approaches to immunotherapy.

Conflicts of Interest

The Authors have no conflicts of interest to declare with respect to this study.

Authors' Contributions

MAS assisted with experimental design, conducted the experiments, and wrote the manuscript. MCH assisted with experimental design, interpretation of results, and writing of the manuscript.

Acknowledgements

These studies were internally funded by the University of Notre Dame (Notre Dame, IN, USA). SIS was contributed by Cook Biotech, Inc. (West Lafayette, IN, USA).

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Received October 12, 2022
Revised October 31, 2022
Accepted November 9, 2022