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Original Article

Effect of a hydrogel-based scaffold material on the establishment of a patient-derived bladder cancer xenograft model

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Abstract: Bladder cancer is treated by surgical removal of the tumor followed by injection of anticancer drugs or the Bacillus Calmette-Guerin vaccine. However, there are insufficient effective drug options depending on the risk category of bladder cancer. One of the reasons for this is the limited number of suitable experimental models that reproduce the pathology of bladder cancer for each risk category. There has been increasing interest in the patient-derived xenograft model as an experimental model to reproduce the original nature of the tumor in a patient. However, there are unresolved problems regarding its practical use, such as the low success rate of engraftment, variation in the growth rate between experiments, and the lack of a reliable method to prepare a patient-derived xenograft model from cryopreserved tumor tissue. In this study, the effect of scaffold material on the preparation of a bladder cancer patient-derived xenograft model was investigated and it was found that gelatin/polyethylene glycol-based hydrogel offers advantages for engraftment of cryopreserved bladder cancer tissue. It was shown that the proliferation of cryopreserved bladder cancer cells was promoted with less necrosis and thrombi around the tissue when transplanted into immunodeficient animals with glycol-based hydrogel compared to transplantation with Matrigel or without any scaffold. This study proposes a new method to generate patient-derived xenograft models from cryopreserved bladder cancer tissue, which is expected to have improved proliferation activity after transplantation.
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Key words: bladder cancer, urinary bladder neoplasms, xenograft, heterografts, PDX, extracellular matrix, hydrogels, cell proliferation, polyethylene glycols, gelatin

Introduction

Bladder cancer is the most common cancer of the urinary tract. Approximately 573,000 people worldwide were diagnosed with bladder cancer in 2020, and this number is expected to increase as the population ages[1]. Approximately 70% of initially diagnosed bladder cancers are non-muscle-invasive bladder cancer (NMIBC), in which the presence of tumor tissue is limited to the mucosal area of the bladder wall. Although NMIBC is treated with trans-urethral resection of the bladder tumor (TURBT) followed by the injection of anticancer drugs or Bacillus Calmette-Guerin (BCG) into the bladder, it frequently recurs and can progress to malignant muscle invasive bladder cancer (MIBC)[2]. Radical cystectomy is often performed once the bladder cancer has progressed to MIBC, resulting in a significant decrease in the patients' quality of life[1, 3]. The development of therapies for bladder cancer has progressed rapidly in recent years, especially new therapies for high-risk bladder cancer patients. However, research is still needed to develop drugs to treat all types of bladder cancer, including NMIBC. Traditionally, anticancer drugs have been discovered through *in vitro* screening tests using patient-derived cancer cell lines or *in vivo* tests using rodents transplanted subcutaneously with a cancer cell line. Many drug candidates have been discovered through these non-clinical experiments, but most have failed to show efficacy in clinical trials. One of the reasons for this is that the original nature of cancer cell lines is compromised by their adaptation to an artificial environment through repeated passages of the cell lines in nutrient-rich media[4]. To overcome this problem, the patient-derived xenograft (PDX) model has recently been developed, in which cancer tissue obtained from patients is transplanted subcutaneously into animals[5]. Although the PDX model is also

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important for bladder cancer drug discovery, few studies have been conducted using this model. It is well known that the difficulty in preparing a PDX model varies from cancer to cancer. In addition, there have been problems to overcome for its practical use, such as the low success rate of engraftment, the variation in growth rate between experiments, and the lack of a reliable method to prepare a PDX model from cryopreserved tumor tissue[6].

Hydrogels are composed of biocompatible polymers, such as collagen, gelatin, and polysaccharides and synthetic materials, such as polyethylene glycol (PEG). They provide permeability for essential molecules (e.g., sugars and proteins) required for cell survival[7]. Gelatin is one of the most widely used scaffold materials for tissue engineering or cell therapy because of its availability, biocompatibility, and good cell attachment[8]. Since gelatin is highly soluble at 37°C and thus, it is difficult to retain the material at the injected site, the solubility can be controlled by properly crosslinking gelatin polymers, while maintaining its bioabsorbable properties[9]. In particular, multi-armed PEG containing N-hydroxysuccinimide ester at the ends (hereafter PEG-NHS) can easily crosslink gelatin polymers at room temperature by chemically bonding lysin residues in gelatin polymers. As this method is also known to be advantageous in terms of cytocompatibility and tissue adhesiveness[10], its use in maintaining cells at the injected site has been investigated[11–13].

We hypothesized that gelatin cross-linked with PEG-NHS (hereafter GP hydrogel), as a cell scaffold material could support the engraftment and growth of patient-derived bladder cancer tissue. The effect of scaffold materials, including GP hydrogel and Matrigel, which is a conventionally used scaffold material, on the growth of cryopreserved PDX tissues after transplantation was evaluated. It was found that the GP hydrogel promoted the growth of transplanted bladder cancer cells, as evidenced by the presence of Ki-67-positive cells and an increase in the volume of transplanted cancer tissues compared to those transplanted with Matrigel or without any scaffold. Histopathological findings after growth showed that necrosis of the transplanted tissues could be better prevented by using scaffolds (GP hydrogel and Matrigel) than by not using scaffolds. In addition, the formation of blood vessels around the transplanted tissue was better promoted by the GP hydrogel than by Matrigel. These results indicate the potential use of GP hydrogel as a scaffold material for establishing a PDX model.

Materials and Methods

Bladder cancer tissue

Bladder cancer tissue used in this study was maintained as TG1 (transplant generation 1) after subcutaneous transplantation into immunodeficient mice at the HOIST Corporation (Osaka, Japan). The characteristics of the bladder cancer tissue are summarized in Table 1. The cancer tissue was transplanted subcutaneously into immunodeficient mice. After sufficient growth, the tissue was removed, cut into pieces, and used for subsequent experiments. This study was approved by the ethics committee of HOIST Corporation.

Table 1. Features of the Bladder Cancer Tissue Used in This Study

Diagnosis	Bladder cancer (Histopathological examination)
Grade	High
Stage	Muscle invasive (T2)
Primary/Recurrence	Recurrence

Animals

C.B-17/IcrHsd-Prkdcscid mice (Japan SLC, Shizuoka, Japan) were obtained at 6 weeks of age and maintained under specific-pathogen-free conditions. Immunodeficient mice were used for the experiments at 7 weeks of age. All experiments and procedures for the care and treatment of the animals used in this study were planned and conducted strictly in accordance with all domestic and international declarations, treaties, laws, regulations, guidelines, or rules, as appropriate. In addition, considerations made for animal welfare and good practice of animal handling with respect to the 3Rs (replacement, refinement and reduction) of animal testing.

Preparation of frozen stock of patient-derived bladder cancer tissue

The TG1 bladder cancer tissue was shipped from HOIST Corporation to Japan SLC at 4°C. The tissue was chopped with a razor blade on ice into blocks of 2 mm^3 and then transplanted subcutaneously into the animals. The transplanted tissues were allowed to grow in the mice to approximately 10 times their original volume and were then removed to obtain TG2 bladder cancer tissues. Some of the TG2 bladder cancer tissues obtained were used for histopathological examination. The remaining tissues were again chopped into blocks of approximately 2 mm^3 and frozen in CELLBANKER® freezing medium (ZENOGEN PHARMA Co. Ltd., Fukushima, Japan) according to the manufacturer's instructions.

Subcutaneous implantation of patient-derived bladder cancer tissue and evaluation of changes in graft size

The frozen TG2 bladder cancer tissues were thawed in a water bath at 37°C on the day of transplantation. They were then cut into pieces of approximately 2 mm^3, immersed in phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA), and rinsed by gentle shaking. After wiping off excess PBS, they were divided into three groups and subjected to different treatments. The first group received no treatment. The second group was immersed in BD Matrigel Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ, USA) and kept on ice until transplantation. The third group was embedded in a self-solidifying hydrogel (GP hydrogel), prepared by cross-linking gelatin with PEG-NHS. The hydrogel was prepared according to

a previously published method[12] with slight modifications. Specifically, APAT high-grade gelatin (Nippi Inc., Tokyo, Japan) was dissolved in D-PBS(-) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) to a concentration of 80 g/L. 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (Dojindo Laboratories, Kumamoto, Japan) was prepared to a concentration of 0.2 mol/L (pH 8.2) using water for injection (Hikari Pharmaceutical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. The 80 g/L gelatin solution was diluted two-fold with the HEPES solution. Separately, four-arm PEG-SC (PEG-NHS, molecular weight 10,000) from SINOPEG (Fujian, China), a tetra-branched PEG with an N-hydroxysuccinimidyl group at the ends, was dissolved in water for injection (Hikari Pharmaceutical Co., Ltd.) to a concentration of 100 g/L. A 1:1 mixture of the 40 g/L gelatin solution and the 100 g/L PEG-NHS solution was applied to coat the tissue pieces before solidification by incubation at room temperature for 5 min. There were four samples in the first and second groups and three samples in the third group. The treated tissue grafts were implanted subcutaneously into mice using a grafting needle (inner diameter, 3.5 mm; length, 85 mm; product number, KN-391-35; Natsume Seisakusho, Tokyo, Japan). No special sutures were applied to the animals. After transplantation, the long and short diameters were measured using a digital caliper (Mitutoyo Corp., Kanagawa, Japan). Based on the lengths obtained, the volume of the graft was calculated as follows:

graft volume (mm^3) = long diameter (mm) × short diameter (mm) × short diameter (mm) × 0.5.

Histopathological examination

On the last day of the experiment, the animals were euthanized and their subcutaneous tumor tissues were collected for histopathological examination. The tissues were fixed in 10% neutral buffered formalin (FUJIFILM Wako Pure Chemical), and hematoxylin and eosin-stained specimens were routinely prepared for histopathological examination. Phosphotungstic acid hematoxylin (PTAH)- and Masson's trichrome (MT)-stained specimens were also prepared using standard procedures. Immunostaining was performed using an anti-Ki67 rabbit antibody (Nichirei Biosciences, Inc., Tokyo, Japan) and Histofine Simple Stain Mouse MAX-PO® (Nichirei Biosciences, Inc.) according to manufacturer's instructions.

Statistical analysis

The percentage of Ki67-positive cells in the tumor tissues was determined by counting the number of Ki67-positive and Ki67-negative cells in five randomly selected microscopic images taken from the viable regions of each tissue group. Images were captured at 20× magnification and included at least 2,000 cells per image, with an average of 3,845 cells.

The number of blood vessels with thrombi was counted in one tissue section for each animal and divided by the area of the viable region of the tissue.

To evaluate the percentage of necrosis in the tumor tissues, the size of the necrotic area and the total tissue area in microscopic images of hematoxylin-eosin-stained tissue sections were quantified using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis was conducted using Dunnett's test to compare changes in the size of the transplanted tissues, the proportion of Ki67-positive cells, the number of blood vessels with thrombi per 1 mm^2 section, and the proportion of necrotic areas in each group relative to the control group (tumor tissue transplanted without scaffold). A p-value <0.05 was considered to indicate statistical significance.

Results

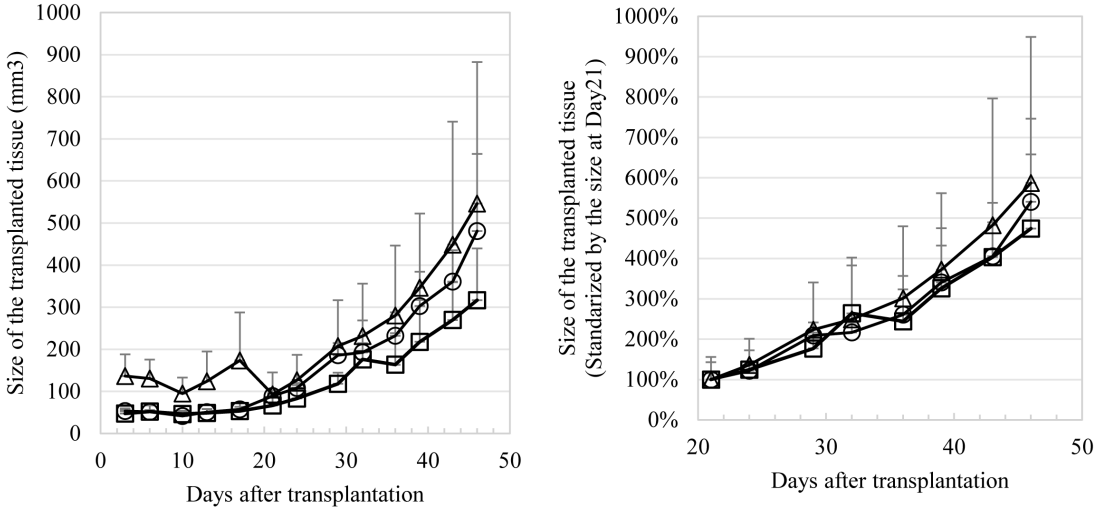
Changes in the size of subcutaneously transplanted patient-derived bladder cancer tissue fragments

The changes in the size of the transplanted cryopreserved TG2 bladder cancer tissues in the animals are shown in Fig. 1A. When transplanted with Matrigel or no treatment, the size of the tissue fragments did not change significantly until day 21 and then increased thereafter. However, when transplanted with GP hydrogel, the size of the tissue fragments decreased until day 21 and then increased rapidly. The initial volume of the transplanted tissue fragments was approximately 2.5 times greater when transplanted with GP hydrogel than when transplanted with Matrigel or no treatment. Therefore, a non-dimensional number was used to indicate the relative grafted volume in Fig. 1B, as the grafted volume at day 21 was defined as 100%, because the volume increase began after day 21. Statistical analysis was performed using Dunnett's test to compare the data obtained, but no statistically significant differences were found between groups. As this experiment was performed with only three or four samples, additional experiments may have been required to show a statistically significant difference. However, on day 46, the size of the tissue fragments increased approximately five-fold with Matrigel and no treatment and six-fold with GP hydrogel. It should be noted that digestion of the GP hydrogel and cell proliferation proceeded simultaneously. Pathological observation on day 46 showed that the GP hydrogel had almost disappeared (Fig. 2). This may suggest that the volume of the grafted tissue was increased more by the GP hydrogel than by the other samples.

Histopathological characteristics of transplanted patient-derived bladder cancer tissue with and without scaffold material

On day 46 after transplantation, tissue fragments were collected from the animals for histopathological examination. The tissue before cryopreservation was also examined for comparison. Representative images are shown in Fig. 3.

Before cryopreservation (A1–A5), the tumor had a poor stromal component and lacked papillary structures and other epithelial-like arrangements. It displayed the high-grade features of human bladder cancer. Tumor cells were



A; The changes of the volume of transplanted tissues

B; The changes of the relative sizes of transplanted tissues standardized at day 21

Fig. 1. Growth of transplanted cancer tissue in immunodeficient mice. A: The changes of the volume of transplanted tissues. B: The changes of the relative sizes of transplanted tissues standardized at day 21. Error bars: standard deviations. Circle(O): Group transplanted without any scaffold material. Square (□): Group transplanted with Matrigel. Triangle (Δ): Group transplanted with GP hydrogel. Each group consists of 4 samples (No treatment and Matrigel) or 3 samples (GP hydrogel).

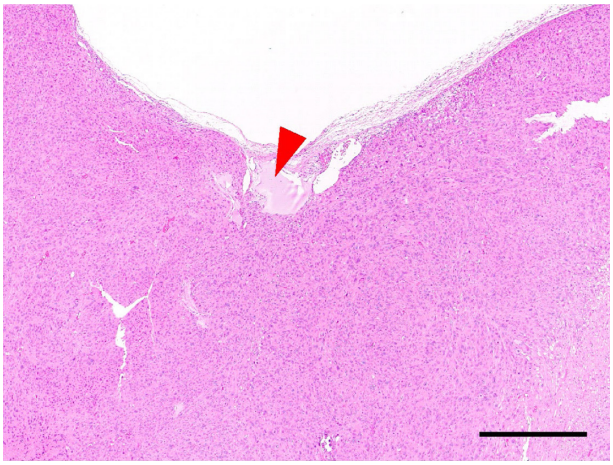


Fig. 2. Residue of GP hydrogel around the cancer tissue after 46 d after hematoxylin and eosin staining. Bar indicates 1,000 μM.

relatively cytoplasmic, with nuclear atypia, a high level of polymorphism, scattered mitotic figures, and a high prevalence of Ki67 positive cells.

In the cryopreserved tissues implanted without the use of a scaffold (B1–B5), necrotic areas were scattered throughout, but prominent areas of necrosis were observed in the center of the tissue. The presence of thrombi suggested a problem with blood supply from the circulation. Tumor cell characteristics and the extent of stroma were similar to those of the tissue before cryopreservation, although fewer Ki67-positive cells were observed.

In the cryopreserved tissues implanted with Matrigel (C1–C5), the characteristics of tumor tissue and cells were maintained. However, thrombi were observed in the tissue, with necrotic cells found around them. Less Ki67-positive cells were observed compared to the cryopreserved tissue implanted without scaffolds.

In the cryopreserved tissues implanted with GP hydrogel (D1–D5), the characteristics of the tumor tissue and cells were maintained. Fewer thrombi and less necrosis were observed, and more Ki67-positive cells were present compared to the cryopreserved tissue implanted with Matrigel. A small amount of hydrogel residue was observed at the periphery, with macrophage infiltration indicating that the degradation process was advancing. No neutrophils or other inflammatory cells were observed.

An attempt at quantitative analysis to compare the characteristics of transplanted cancer tissue

Figure 4 shows the percentage of Ki67-positive cells observed in tissue sections for each group, while Fig. 5 presents the number of blood vessels with thrombi per 1 mm^2 of viable tissue. Statistical analysis was conducted using Dunnett's test to compare the percentage of Ki67-positive cells and the number of blood vessels with thrombi per 1 mm^2 of viable tissue in each group to the group in which cryopreserved cancer tissues were transplanted without scaffold material. In this analysis, Ki67 positivity was statistically different in the tissue with the GP hydrogel than in the other tissues.

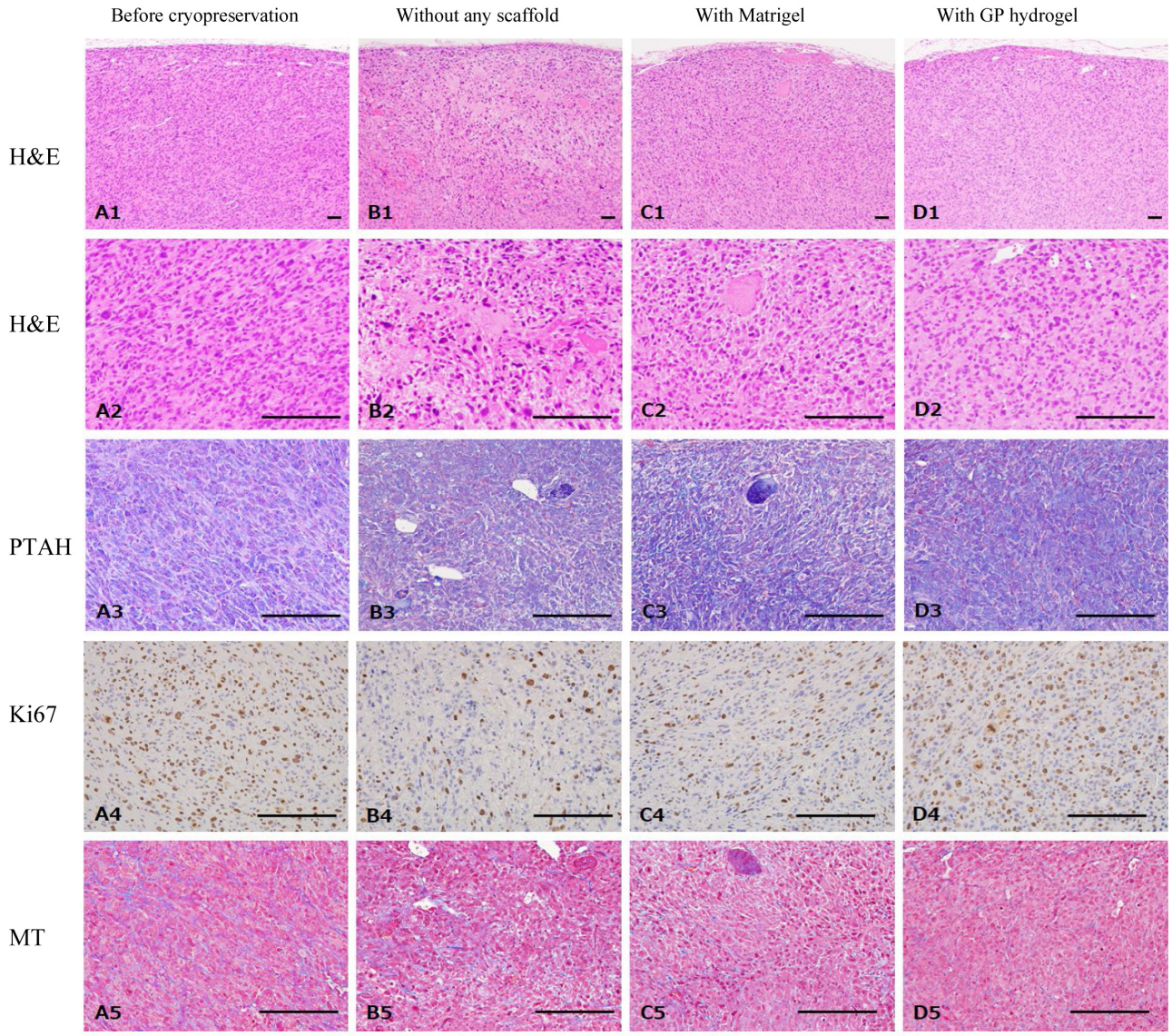


Fig. 3. Representative images of cancer tissues before cryopreservation and 46 d after transplantation, with and without scaffold material. A1–A5: Before cryopreservation. B1–B5: Transplantation without any scaffold material. C1–C5: Transplantation with Matrigel. D1–D5: Transplantation with GP hydrogel. A1–D1, A2–D2: Hematoxylin and eosin staining. A3–D3: PTAH. A4–D4: Ki67. A5–D5: Masson's trichrome staining. Bars indicate 200 μM.

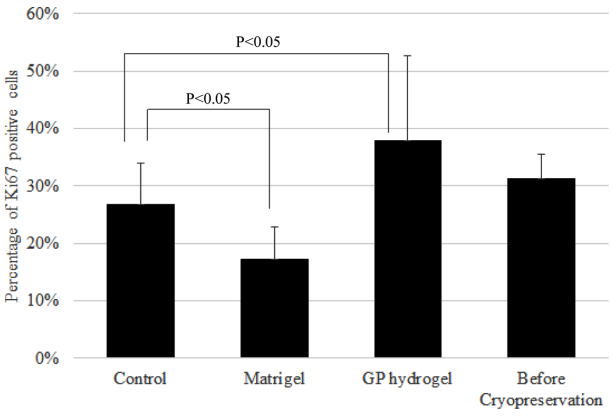


Fig. 4. Comparison of percentage of Ki67-positive cells at 46 d after transplantation, with and without scaffold material, and before cryopreservation. Error bars: standard deviation. Each group consists of four samples (no treatment and Matrigel), three samples (GP hydrogel), or one sample (before cryopreservation). Line segments represent statistical significance calculated using Dunnet's test.

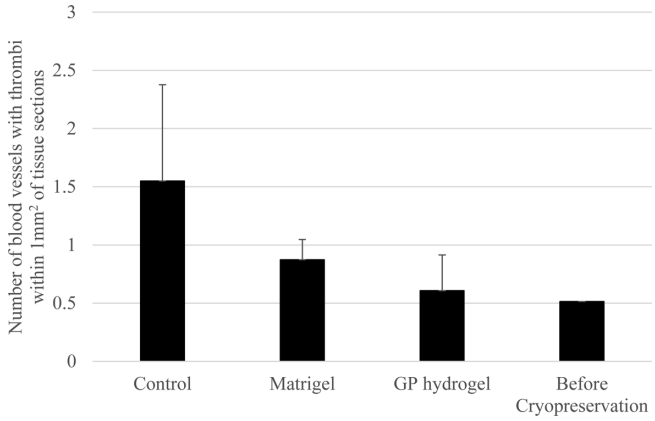


Fig. 5. Comparison of the number of blood vessels with thrombi counted in tissue sections obtained 46 d after transplantation, with and without scaffold material, and before cryopreservation. Error bars: standard deviation. Each group consists of four samples (no treatment and Matrigel), three samples (GP hydrogel), or one sample (before cryopreservation).

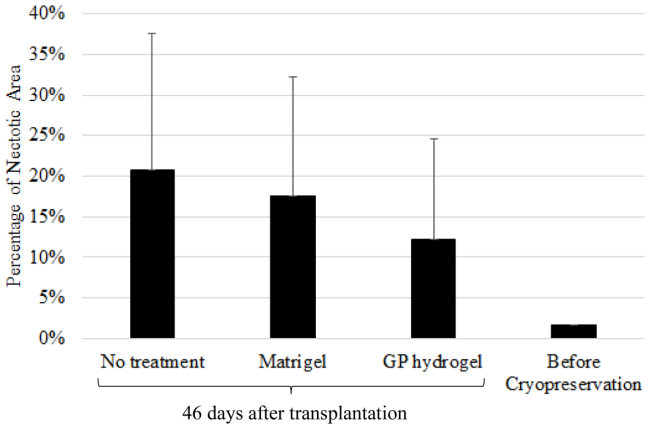


Fig. 6. Comparison of percentage of necrotic area at 46 d after transplantation, with and without scaffold material, and before cryopreservation. Error bars: standard deviation. Each group consists of four samples (no treatment and Matrigel), three samples (GP hydrogel), or one sample (before cryopreservation).

Proportion of necrotic area in the transplanted patient-derived bladder cancer tissue, with and without scaffold material

The proportions of necrotic areas observed in the center of the transplanted tissue are shown in Fig. 6. The necrotic area decreased when the cryopreserved cancer tissue was transplanted with the GP hydrogel compared to when the tissue was transplanted with Matrigel or without a scaffold. A smaller necrotic area was observed in the tissue transplanted with Matrigel compared to that transplanted without any scaffold. Statistical analysis was performed using Dunnett's test, but no statistically significant differences were found between groups. As this experiment was performed with three or four samples, an additional number of experiments may have been required to show a statistically significant difference. However, the results suggested that the GP hydrogel reduced the amount of necrosis in the cryopreserved cancer tissues when transplanted subcutaneously into animals.

Discussion

In this study, we investigated the effect of a GP hydrogel on the establishment of a PDX model from cryopreserved cancer tissues. As shown in Fig. 1, the size of subcutaneously transplanted tissue in immunodeficient mice showed the highest rate of increase when the GP hydrogel was used. Histopathological examination revealed the presence of necrotic areas when cryopreserved tumor tissue was transplanted without scaffold material (B1 and B2 in Fig. 3), with necrosis more prevalent at the center of the tumor tissue than at the periphery. The presence of thrombi suggested that necrosis was likely induced in areas with insufficient blood supply. Consistent with known PDX model characteristics, central necrosis was observed in the tumor tissue. In the area that remained viable, the tumor tissue arrangement and tumor cell characteristics were similar to those of the tissue before cryopreservation.

However, decreased necrosis of the tumor tissue and improved proliferative activity were observed when transplanted with scaffold material. In the tissue transplanted with the GP hydrogel, less necrosis and fewer thrombi and more Ki67-positive cells were observed than in the tissue transplanted with Matrigel. We speculate that these differences were advantageous for tumor growth in the group transplanted with the GP hydrogel, as observed in the size measurements of the tumor tissue. The characteristics of the tumor tissues were similar to the characteristics before cryopreservation when the scaffold materials, Matrigel, or GP hydrogel were used. Different features, such as an increase in stromal components, were not observed. Although several aspects, such as tissue growth, reduced necrosis, and decreased thrombus formation, were observed with the GP hydrogel, a statistically significant difference was found only in the percentage of Ki67-positive cells. Further investigation with a larger sample size is needed to clarify the specific contributions of the GP hydrogel.

Gelatin in the GP hydrogel is a well-known material with excellent cell adhesion properties, and this may have promoted the adhesion of the transplanted tumor cells to the surrounding tissues and earlier initiation of angiogenesis and anastomosis, leading to improved nutrient supply from the blood flow around the transplanted tissues. The relationship with the surrounding tissues remains to be elucidated in future studies. It is known that the GP hydrogel degrades *in vivo* and thus, does not affect the growth of tumor tissue (data not shown). Although residues of the material were observed around the tumor tissue 46 d after transplantation, the amount was small enough that it did not affect the size of the tumor tissues. Mild infiltration of macrophages was

observed, suggesting that the degradation process was ongoing.

In summary, the possibility of using a GP hydrogel as a novel scaffold material to support the establishment and passaging of PDX models was investigated in this study. Although Matrigel is a well-known scaffold material used to establish PDX models, it has been noted that it is not optimal for a PDX model for bladder cancer[6]. Therefore, it is worthwhile to explore alternative scaffold materials. GP hydrogel is potentially an excellent scaffold to establish such a model, especially when using cryopreserved tumor tissues. It has also been shown that GP hydrogels degrade *in vivo* and do not affect the morphology of tumor tissues growing *in vivo*. Although we investigated the effect of a GP hydrogel on bladder cancer in this study, these findings may be applicable to other cancers. We propose a new methodology to establish PDX models with improved survival and proliferation of tumor cells, especially when using cryopreserved tumor tissue prepared through multiple passages in animals. We believe that further investigation of the maintenance of genetic characteristics and the relationship with the surrounding tissues after transplantation (especially in terms of angiogenesis and the maintenance of circulation) will elucidate the characteristics of the GP hydrogel more clearly in the future.

Disclosure of Potential Conflicts of Interest: Takeshi Yamamoto and Hayato Miyoshi are employees of FUJIFILM Corporation, Japan. Shinji Mima and Chihaya Kakinuma are employees of HOIST Corporation, Japan. Hiroyuki Kamata is an employee of Gellycle Co., Ltd., Japan. Yuji Nozaki and Hisayoshi Takagi are employees of Japan SLC, Inc., Japan

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