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Engineering fibrotic tissue in pancreatic cancer: A novel three-dimensional model to investigate nanoparticle delivery

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ABSTRACT

Pancreatic cancer contains both fibrotic tissue and tumor cells with embedded vasculature. Therefore anti-cancer nanoparticles need to extravasate from tumor vasculature and permeate thick fibrotic tissue to target tumor cells. To date, permeation of drugs has been investigated *in vitro* using monolayer models. Since three-dimensional migration of nanoparticles cannot be analyzed in a monolayer model, we established a novel, three-dimensional, multilayered, *in vitro* model of tumor fibrotic tissue, using our hierarchical cell manipulation technique with K643f fibroblasts derived from a murine pancreatic tumor model. NIH3T3 normal fibroblasts were used in comparison. We analyzed the size-dependent effect of nanoparticles on permeation in this experimental model using fluorescent dextran molecules of different molecular weights. The system revealed permeation decreased as number of layers of cultured cells increased, or as molecule size increased. Furthermore, we showed changes in permeation depended on the source of the fibroblasts. Observations of this sort cannot be made in conventional monolayer culture systems. Thus our novel technique provides a promising *in vitro* means to investigate permeation of nanoparticles in fibrotic tissue, when both type and number of fibroblasts can be regulated.

1. Introduction

Pancreatic cancer is a devastating illness. The median survival time of patients with advanced pancreatic adenocarcinoma is approximately 6 months despite recent progress with conventional chemotherapies [1]. Although cancer cells derived from these tumors, cultured two dimensionally *in vitro*, are sensitive to anti-cancer agents, most fail to inhibit tumor growth *in vivo*. For example, in an *in vitro*, two dimensional model of pancreatic cancer, using BxPC3 cells, gemcitabine exhibits potency [2], but the same is not true of a xenograft *in vivo* [3].

A major obstacle to efficacious drug dose may be insufficient drug delivery to tumor cells due to hypovascularity and dense fibrosis [4,5]. Therapeutic drugs, even if they succeed in migrating from tumor blood vessels, have to permeate interstitium to reach tumor cells. This problem is especially pertinent in the case of pancreatic cancer because the interstitium is mainly fibrotic tissue including activated fibroblasts [6–8]. Despite this acknowledged problem there is, to date, no satisfactory modeling of drug permeability in fibrotic tissue. The problem is further complicated by emerging nano drug delivery systems (nanoDDS) [9], in which molecule size is larger than conventional anti-tumor agents of MW ~ 350. This increased size may present additional extravasation [10] and permeation problems in tumor fibrotic tissue.

Permeation of drugs, including nanoparticles, in tissues has been investigated *in vitro* using endothelial cells or epithelial cells cultured in monolayer [11,12], but three-dimensional migration of the drugs cannot be analyzed in monolayer systems. On the other hand, many three-dimensional models have been used to mimic cells including fibroblasts in environments *in vivo* [13–17], but they have not addressed the problem of drug permeability. For example, collagen gel is widely used because it resembles extracellular matrix (ECM), but it shrinks after a number of days of cell incubation so cannot be used to test permeability. If it were possible to make an artificial three-dimensional model of the fibrotic components of pancreatic cancer it might be possible to observe

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extracellular diffusion of nanoparticles using fluorescent markers *in vitro*. Recently, we reported an *in vitro* hierarchical, cell manipulation technique to develop three-dimensional cellular multilayers via fabrication of nanometer-size extracellular matrix (ECM) films on the surface of each cell layer [18–21]. Approximately 6 nm thick fibronectin–gelatin layer-by-layer films fixed to the surface of a first layer of cells provides a cell-adhesive surface, similar to natural ECM, for a second layer of cells.

Here we provide quantitative analysis of diffusion of nanoparticle, dextran molecules in an *in vitro* three-dimensional model of fibrotic tissue in pancreatic cancer. The new model was established using fibroblasts derived from pancreatic tumor, K643f, in comparison with NIH3T3 normal fibroblasts. It revealed that permeation decreased as number of cell layers increased, or as molecule size increased. In addition the source of the fibroblasts was shown to affect permeation. These factors cannot be analyzed in conventional, monolayer, experimental systems. Thus our three-dimensional model will be useful for analyzing permeation of drugs, including nanoparticles, for diseases such as pancreatic cancer.

2. Materials and methods

2.1. Cell culture

K643f fibroblasts were established from spontaneous murine models of pancreatic tumor *in situ* as described previously [22,23]. K643f cells were cultured in RPMI 1640 (GIBCO Life-technologies, Paisley, UK) supplemented with 20% FBS and 1% penicillin/streptomycin. NIH3T3 fibroblasts were obtained from ATCC (Rockville, MD, USA) and cultured in DMEM (GIBCO) supplemented with 10% FBS and 1% penicillin/streptomycin (GIBCO).

2.2. Fabrication of cellular multilayers

Cellular multilayers were fabricated on transwell inserts (BD Falcon) using the layer-by-layer technique described previously [18]. Briefly, 1.44×10^5 cells were seeded on the cell-culture insert and incubated in Dulbecco's modified Eagle medium (DMEM) with 10% FBS for 12 h at 37 °C. The monolayer cells on the substrate were alternately immersed into 0.2 mg/ml fibronectin (Sigma, St. Louis, MO, USA) or gelatin (Wako, Osaka, Japan) solution (50 mM Tris–HCl buffer, pH 7.4) seven times, and rinsed with 50 mM Tris–HCl buffer for 1 min each at 37 °C. This provides an adhesive scaffold for a second layer of cells. 1, 2, or 5 layers of fibroblasts were built at the rate of one layer per day. To assure cellular adhesion, each layer was prepared more than 12 h after formation of the previous layer.

2.3. Dextran-permeation test

FITC-conjugated dextran of 250 kDa, 500 kDa, and 2000 kDa (or 2 MDa) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The hydrodynamic diameter of dextran was measured by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments, UK). Dextran was dissolved in the cell culture medium and added to the upper chamber of the insert, after brief cell washing, a day after preparation of the final layer. The medium in the lower chamber was collected at given time points and fluorescent intensity measured by Nanodrop (ND-3300; Thermo Scientific, Wilmington, DE, USA). The medium was briefly stirred before sampling and a given amount of upper-chamber medium taken immediately to maintain the same surface as the lower chamber. Permeation percentage was determined using the equation below:

$$\begin{aligned}\text{Permeation}(\%) = ((&\text{concentration in the lower chamber}) \\ &\times (\text{lower volume}) / (\text{initial concentration in the} \\ &\times \text{upper chamber}) \times (\text{upper volume})) \times 100.\end{aligned}$$

2.4. H&E staining

The cells cultured in multilayers were snap-frozen in OCT compound (Sakura Fineteck, Tokyo, Japan) to make cryosections. They were then sectioned in 4 μm slices using a cryotome (Cryostat 1800, Leica), fixed with formalin (Wako), and stained with hematoxylin and eosin. Slides of the slices were observed by microscope (DP20, Olympus) with ×10 objective lens.

2.5. Phalloidin staining

Phalloidin staining was used to compare cellular structure as cultured in gelatin with multi-layers. Both gelatin and multi-layers were fixed with formalin (Wako, Osaka, Japan), washed with PBS, blocked with BlockingOne (Nacalai tesque, Kyoto, Japan) and incubated overnight with Phalloidin (Sigma–Aldrich, St. Louis, MO) diluted 1:100. We mounted the samples on glass slides and observed them using confocal microscopy (LSM510 META, Zeiss).

2.6. Pathological specimens of human pancreatic cancer

The specimens of pancreatic cancer were taken from patients in Hokkaido University undergoing surgical resection of primary foci. Written blanket informed consent was obtained from the patients, and the experiment was approved by the Ethics Committee of Hokkaido University. The specimens were fixed with formalin and embedded in paraffin. Thin sections of the paraffin block were stained with hematoxylin and eosin (H&E staining).

2.7. Statistical analysis

Statistical analysis, where applicable, was carried out using Excel software (Microsoft, Redmond, WA, USA). Results were compared using Student's *t*-test and expressed as mean values with standard deviations (SD). Differences were considered statistically significant at $p < 0.05$.

3. Results

We first observed H&E-stained samples of human pancreatic cancer to observe their structure as a guide to making a mimetic model using the multilayered cell culture technique. Histologically, human pancreatic tissue, aside from tumor cells, reveals a large volume of fibrotic tissue with occasional vasculature distal to the tumor cells (Fig. 1A). Drugs administered to patients, to reach the disease site, must pass through vasculature and fibrotic tissue to target cells [5,24]. For this reason understanding migration through fibrotic tissue is important especially with nanoDDS, which have a molecular diameter (or hydrodynamic diameter) far larger than conventional drugs. Multilayering of K643f cells, a cell line of fibroblasts derived from a murine spontaneous pancreatic tumor model, was engineered on a culture insert (Fig. 1B). We confirmed the integrity of the structure by transmission light microscopy plus microscopic observation of phalloidine- and H&E-stained samples (Fig. 1C–E). We observed an ordered, stable and firm structure. The thickness of the multilayer was 30–50 μm with 5 layers of cultured cells.

Next we observed permeation of modeled nanoparticles, via fluorescence-labeled dextran of large molecular weights, through the multilayer system to investigate delivery of nanoDDS to tumor fibro-

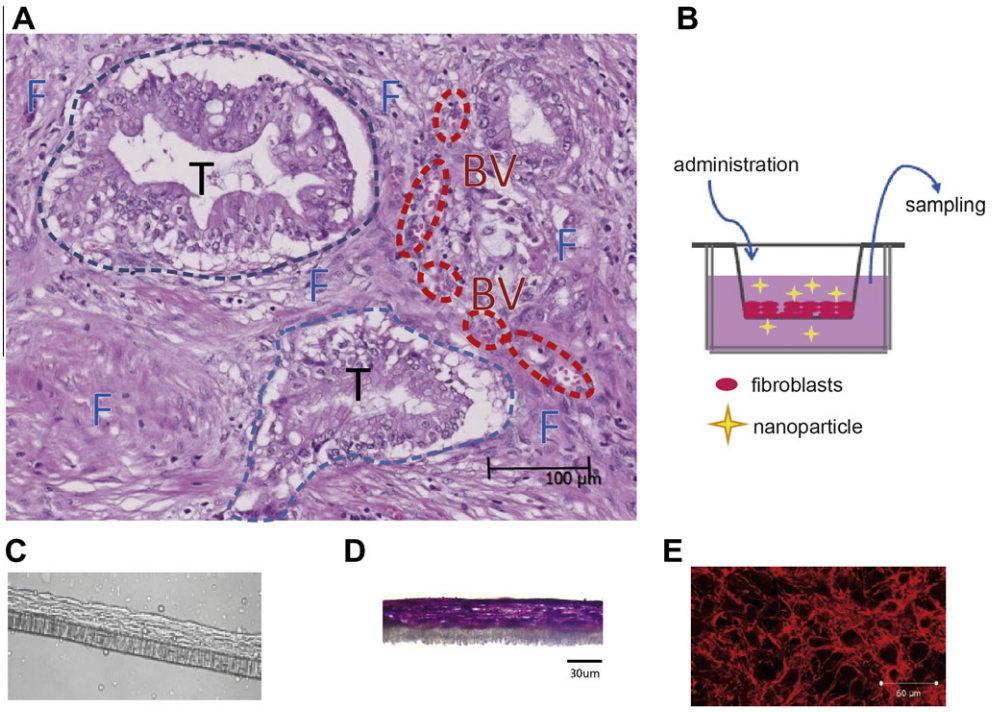

Fig. 1. Histological pattern of human pancreatic cancer and the structure of the model. (A) H&E staining of a pathological specimen of human pancreatic cancer. Note that a large part of the tumor tissue is occupied by fibrotic tissue. T, tumor cells (inside blue dotted circles), BV, blood vessels (inside red dotted circles), and F, fibrotic tissue (other parts). (B) A scheme of the model. We used the transwell insert to establish a multilayered culture of fibroblasts. We administrated nanoparticles into the culture media on the transwell, and measured the concentration of fluorescence in the media below the transwell. (C) Transmitted light microscopy of the multilayered culture. (D) H&E staining of the multilayered culture. (E) Phalloidin staining of the multilayered culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

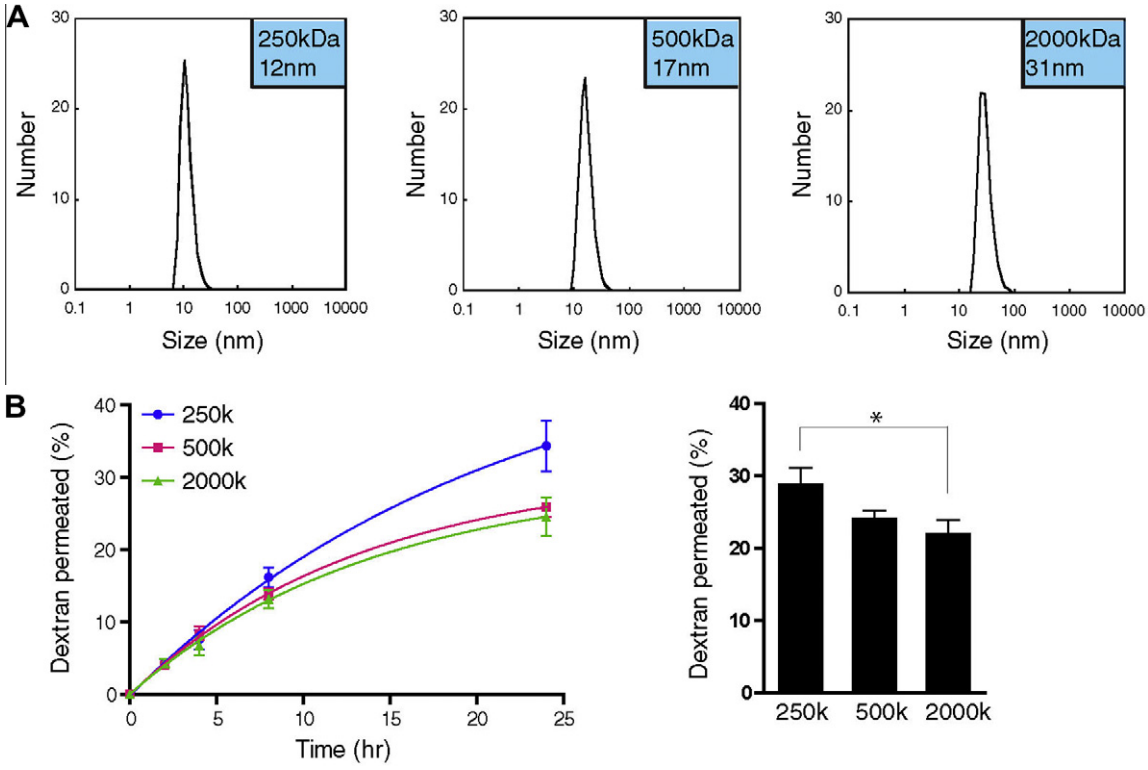

Fig. 2. Dextran as the modelled nanoparticle and its permeation. (A) Measurement of hyrodynamic diameter of FITC-conjugated dextran molecules of 250 kDa, 500 kDa, or 2000 kDa was done by dynamic light scattering. (B) Permeation of dextran was followed in the monolayer model for 24 h using K643f cells (left). Percent of permeation at 24 h was compared between molecular sizes (right). Bars indicate SD, *Represents $p < 0.05$.

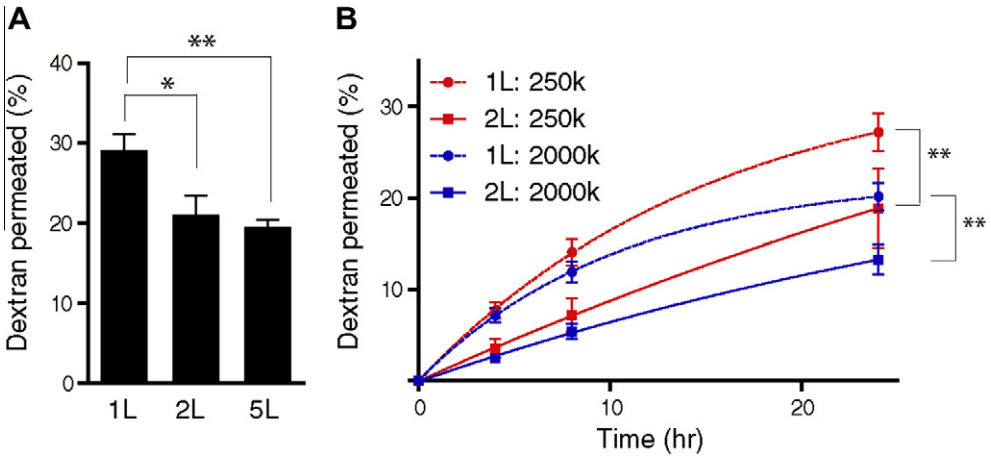

Fig. 3. Number of layers and pearmeation of dextran. (A) Dextran of 250 kDa was tested for permeation in 1, 2, and 5 layers (L) of K643f multilayered culture. Results at 24 h are shown. Bars, SD, *Represents $p < 0.05$, **Represents $p < 0.01$. (B) Dextrans of 250 kDa or 2000 kDa were tested in 1 or 2 layers of K643f culture. Bars, SD.

tic tissue. We first measured the hydrodynamic diameter of dextran with different molecular weights (250 kDa, 500 kDa, or 2000 kDa) by DLS. The hydrodynamic diameters were: 12, 17, and 31 nm, respectively (Fig. 2A). Permeability of dextrans was first tested by measuring fluorescence in a monolayer culture of K643f cells. The dextran of 2000 kDa showed significantly less permeability compared to 250 kDa and 500 kDa (Fig. 2B). At 24 h the permation percentages were: 250 kDa = 28.8 ± 8.5, 500 kDa = 24.1 ± 3.5 ($p = 0.090$ vs. 250 kDa), and 2000 kDa = 22.0 ± 6.0 ($p = 0.036$ vs. 250 kDa, but not significantly different when compared to 500 kDa, $p = 0.37$). Therefore we used dextran of 250 kDa and 2000 kDa for comparison.

We then measured the permeability of the dextrans in K643f multilayer cultures of 1, 2, and 5 layers. Dextran of 250 kDa permeated culture systems with more layers less well. Permeability was: 28.8 ± 8.5% in 1 layer, 21.1 ± 5.7% in 2 layers, and 19.2 ± 2.4% in 5 layers (1 layer vs. 2 layers: $p = 0.035$, 1 layer vs. 5 layers: $p = 0.0024$, Fig. 3A). Permeability through 2 and 5 layers was not significantly different ($p = 0.50$). Therefore, we used K643f cultured in 2 layers as the multilayer culture. Dextran permeability of 2000 kDa was then compared in K643f cells cultured in single layer or double layer. In this model, the number of layers was the major determinant of permeability.

Finally we tested multilayered cultures of NIH3T3 cells (Fig. 4A) using normal fibroblasts in comparison with fibroblasts derived from tumor K643f. We tested dextran permeability of 250 kDa or 2000 kDa. We found the NIH3T3 model exhibited a slightly different pattern of permeation (Fig. 4B). Although the reason for this remained unclear in the NIH3T3 model, permeability decreased with increased molecule size, and with increased layer number in the K643f model (Fig. 3B).

4. Discussion

Interstitial tissue is a major problem for nanoDDS delivery especially with pancreatic cancer where fibrotic tissue is relatively dense. Investigation of this problem requires a reliable three-dimensional culture model that mimics tumor fibrosis, and can be used to assess drug permeability. We cannot analyze three-dimensional migration of nanoparticles using a conventional, two-dimensional monolayer culture system. A previous three-dimensional model *in vitro* using collagen gel mixed with fibroblasts was found ineffective for the purpose, since it showed low cell density and the gel shrank following several days of culture (Hosoya et al., unpublished observation, 2011). In contrast to *in vitro* systems, building *in vivo* three-dimensional animal cancer models with large amounts of fibrotic tissue to resemble human cancer is difficult [25]. A mouse tumor model using BxPC3 human-derived pancreatic cancer cells, which we used as a stroma-rich pancreatic cancer model [5,24,26], nevertheless possessed lower fibrotic tissue volume than in humans. So far as we know there is currently no way of accurately managing the amount of fibrotic tissue in animal tumor models.

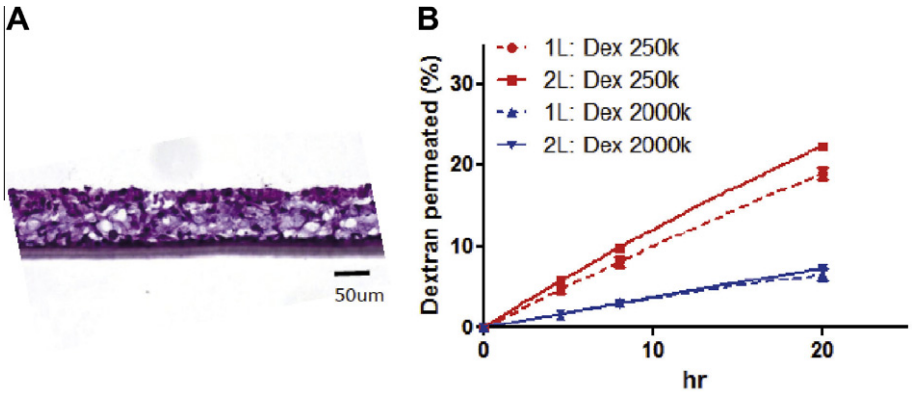

Fig. 4. Multilayered culture of NIH3T3 cells as normal fibroblasts. (A) H&E staining of the multilayered culture of NIH3T3 cells. (B) Dextrans of 250 kDa or 2000 kDa were tested for permeation in multilayered NIH3T3 culture. Bars, SD, ****Represents $p < 0.0001$.

Here we have taken the more manageable route of fabricating a multilayer model of fibroblasts on a culture insert using the layer-by-layer technique [16]. And we were able to show *in vitro* that permeation of nanoparticles was dependent either (1) on the numbers of layers of cultured cells, or (2) on the molecule sizes of the nanoparticles. Our new model showed a well-ordered structure, as confirmed by both H&E and Phalloidin staining, that mimicked the structure of interstitial tissue in human pancreatic tumor. It was thus possible to test nanoparticle permeability. In addition, this multilayered culture technique enables us to regulate the amount and types of fibroblasts in a model, and offers a way to test drug permeability for various tumor interstitial tissues including that of pancreatic cancer.

We observed that increasing the number of layers in our new model affected fibroblasts from pancreatic tumor (K643f) more than normal fibroblasts (NIH3T3). This may mimic the situation in which tumor interstitial tissue become less permeable to drugs the more it becomes fibrotic. Characteristic differences between K643f, a cell line of fibroblasts derived from tumor, and NIH3T3, a normal fibroblast cell line, are of interest: one difference may be connected with the ECMs produced by these two cell lines [7]. These are secreted around fibroblasts and may block the migration and permeation of nanoparticles. When compared to normal fibroblasts including NIH3T3, activated fibroblasts in tumors, including cancer-associated fibroblasts (CAFs), are known to exhibit enhanced secretion of ECM proteins, such as type I collagen, tenascin C, and fibronectin, as well as proteases degrading ECM. Junction formation between cells may also be another factor in play. Such intercellular junctions may not only be anatomically narrower, but also alter signal transduction. This may affect cellular responses including ECM production [27,28]. Analysis of these multiple factors, using different cell lines including CAFs, and normal fibroblasts, is a future study. Because K643f was established from activated-Kras mouse pancreas, which contains only murine pancreatic intraepithelial neoplasia (mPanIN) tissue, the cell line is not a true model of CAFs. Nevertheless, our new model enables analysis of a sort previously unavailable.

Permeation testing of Dextran of different molecular weights [29] showed that increase in layer number decreased permeability, and that increased molecular weight decreased permeability. This suggests that molecular weight of a drug affects permeability because of intersitial tissue. We can thus hypothesize that ECM produced from fibroblasts may form pores of a certain size for the nanoparticles, through which the dextran permeates. To imagine how this works we can compare it to electrophoresis using gels, where electrically charged molecules migrate a given distance relative to their size. In this situation blood pressure may be the driving force in tumor tissues *in vivo* rather than convection, as previously reported [30], although this aspect of molecular migration remains to be elucidated.

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