Pericyte-Coverage of Human Tumor Vasculature and Nanoparticle Permeability

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Nano drug delivery systems (nanoDDS) are a promising strategy for treatment of human tumors. As indicated by our previous work, the extent of pericyte-coverage of tumor vasculature is important to determining nanoDDS efficacy since intratumoral accumulation of nanoDDS is less in tumor models with pericyte-covered vasculature. Here we investigated the clinical relevance of our previous observations in animal models, by determining pericyte coverage using immunohistochemistry of smooth muscle actin (SMA) with CD34: a vascular endothelial marker, in human tumor tissue samples. The investigation revealed that tumor vasculature coverage by pericytes in pancreatic and diffuse-type gastric cancers was significantly greater than in ovarian, colon, and intestinal-type gastric cancers. The latter group of cancers is easier to treat clinically. These observations are consistent with our previous findings in animal models. On the basis of these findings we believe optimization of nanoDDS delivery should be done depend upon a clear understanding of the effects of pericyte vascular coverage.

Key words nano drug delivery system; refractory tumor; tumor vasculature; pericyte; desmoplasia

Efficacious treatment of solid tumors using nano drugs is an important goal of chemotherapy. However, clinically approved nano drug delivery systems (nanoDDS) have thus far shown poor performance in all but a few tumors. On the other hand, they do look effective in xenograft models. For example, Doxil, the PEGylated liposome incorporating doxorubicin, has been approved for clinical use in treatment of Kaposi’s sarcoma and ovarian cancer. Kaposi’s sarcoma, common in untreated human immunodeficiency virus (HIV) individuals, consists mainly of vascular endothelial cells. The tissue of the tumor also has abundant vascular space. Similarly, widely-used xenograft tumors in animals tend to have abundant vascular space. The aim of the present research is to help improve efficacy of nanoDDS in difficult cancers such as pancreas.

Drug delivery to tumor tissue determines treatment efficacy. For example, Gemcitabine, the first-line anticancer agent for pancreatic adenocarcinoma, exhibits potent in vitro inhibitory effects on cells derived from the human pancreatic adenocarcinoma line BxPC3. However, its inhibition of xenograft BxPC3 tumors in mice is unimpressive. And in humans, Gemcitabine only prolongs survival, with significant effects confined to improvement of quality of life.

Tumor vasculature may explain the difference in effects: particularly with reference to drug delivery. Extravasation of drugs to tumor tissue is essential to drug migration, with drug molecule size another determinant of accumulation especially for nanoDDS. Our hypothesis is that pericytes play an important role in determining extravasation. To identify tumor pericytes in this work, we used alpha smooth muscle actin (SMA). SMA is a commonly used marker and readily detected in tumor vasculature pericytes both in humans and mice, although it is often absent from pericytes in normal tissue. We used CD34 as the marker for human vascular endothelial cells.

Previously, we compared xenografts of pancreatic cancer and diffuse-type gastric cancer, both devastating in humans, with that of colon cancer, which is more amenable to treatment. BxPC3 human-derived pancreatic cancer cells, OCUM-2MLN human-derived diffuse-type gastric cancer cells, and C26 murine colon cancer cells, were used in the studies. C26 xenograft is a familiar model for a range of human cancers, especially in connection with nanoDDS. However, compared to C26 xenografts, BxPC3 and OCUM-2MLN xenografts, in connection with nanoDDS, were devastating even in animal models. The histology of BxPC3 and OCUM-2MLN xenografts revealed that they were stroma-rich and more fibrotic. In addition, the vasculature of these xenografts was more covered by pericytes. Based on these observations in animals, we hypothesize that 1) stroma-rich tumors have more pericyte coverage, and 2) pericyte coverage determines extravasation of nanoDDS. For these reasons stroma-rich tumors are devastating even for nanoDDS. Although it is not easy to validate the second part of our hypothesis in humans, because we would need clinical approval for nano-tracer use, we can check the first part of the hypothesis using pathological samples.

To elucidate 1), we analyzed pericyte coverage of tumor vasculature in human surgical samples of ovarian, pancreatic, gastric, and colon cancers.

MATERIALS AND METHODS

Human Histopathology Samples We examined surgical samples from five patients with ovarian, pancreatic, colon, or gastric cancer. The patients all came from Hokkaido University Hospital, Japan. The samples were obtained under blanket, written, informed consent, and the experiment approved by the Ethics Committee of Hokkaido University. Tissue sections were prepared from paraffin blocks and stained with Hematoxylin and Eosin (HE). Immunohistochemical analysis was performed using primary antibodies for CD34

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(Nichirei Bioscience Inc., Tokyo, Japan), and smooth muscle actin (DAKO Japan Co., Kyoto, Japan), followed by antibody detection with a peroxidase-conjugated streptavidin-dimethylaminoazobenzene (DAB) readout system (DAKO). Samples were observed with an Olympus AX80 microscope (Tokyo, Japan).

**Animal Cancer Models**

Xenografts using BxPC3 human pancreatic adenocarcinoma cell line or the murine colon adenocarcinoma C26 cell line were established as previously described. Briefly: 5×10⁶ BxPC3 cells or 1×10⁶ C26 cells were implanted by subcutaneous injection into the abdominal region of BALB/c nude and BALB/c normal mice. They were then allowed to grow until they proliferated for 3 weeks and 1 week, respectively. All experimental protocols were carried...
out in accordance with the policies of the animal ethics committee in the University of Tokyo. Excised samples were fixed overnight in 4% paraformaldehyde then paraffin embedded to prepare them for HE staining. Samples were observed with an AX80 microscope (Olympus, Tokyo, Japan).

RESULTS

We first observed the vascular structure in cases of human ovarian cancer (Fig. 1). Ovarian cancer is one of indications of treatment with Doxil, a nanoDDS already approved for clinical use. The tumor neovasculature was sparsely covered by SMA-positive pericytes, confirming our observation of nanoDDS-treatable tumor neovasculature in animals.

In our previous animal models of colon cancer and pancreatic cancer, the former was seen to possess fewer pericytes and the latter more. Accordingly, the colon cancer model had better distribution of nanoparticles, while the pancreatic cancer showed less, and was difficult to treat with nanoDDS alone. Human histopathology specimens revealed similar pattern of pericyte-coverage (Fig. 2). The vasculature in pancreatic cancer was densely surrounded with SMA-positive cells including pericytes. However in colon cancer, the vasculature was both less associated with SMA-positive pericytes and more pronounced. The abundant SMA-positive cells, especially in pancreatic cancer, and aside from perivascular cells in stroma, were thought to be myofibroblasts or activated fibroblasts associated with desmoplasia.

We further examined the specimens of human gastric cancers from the same viewpoint (Fig. 3). Here we separated the samples into two types according to the Laurén classification: an intestinal type and a diffuse type. Diffuse-type, or scirrhous, gastric cancer is known to be a far more devastating disease than intestinal-type gastric cancer.

Immunohistochemical analysis of these two types of gastric cancer showed that the vasculature of the diffuse-type had more SMA-positive pericytes than the intestinal-type.

Figure 4, that includes a schematic representation of nanoDDS delivery into tumor, summarizes our observations of human samples in combination with the histology of two representative animal xenografts. Vasculature tightly packed with pericytes may part block extravasation of nanoDDS (Fig. 4, right column). Here we describe this phenotype as: “tight vasculature,” and BxPC3 xenograft may be regarded as representative of this phenotype, where vasculature (arrowheads) shows mature wall structure around the lumen containing red blood cells. Human pancreatic and diffuse-type gastric cancers were classed as belonging to this “tight vasculature” category. On the other hand, vasculature less tightly packed with pericytes was unstable and leaky: a common tumor vasculature phenotype. This “leaky vasculature” allows nanoDDS extravasation. C26 xenograft may be regarded as a representative model of this phenotype.

Fig. 3. Serial Sections of Surgical Samples from Human Gastric Cancer, Intestinal and Diffuse Types: HE-Stained or Immunostained against CD34 and SMA

Red arrows (in online journal): vasculature. Bars, 50 μm. Inset: a representative image of vasculature at higher magnification. Bars, 10 μm.
not present a wall-like structure on HE staining, although it does possess endothelial cells. Human ovarian, colon, and intestinal-type gastric cancer all belong in this category.

DISCUSSION

Following our previous studies using animal tumor models, the extent of tumor vasculature coverage by pericytes affects intratumoral accumulation of nanoDDS. Therefore, it is important to understand to what extent the animal models are useful homologs of human cancer.

As to markers used to identify pericytes, we used several beside SMA in this study. These include: NG2 and PDGFRβ. According to the immunostaining of our tumor samples, NG2 was negative and PDGFRβ was positive for broader cell populations including perivascular cells (Kano and Nishihara et al., unpublished observations). For this reason we did not use them in our study. SMA, however, is also positive for myofibroblasts or activated fibroblasts in tumor stroma. Therefore, in this work we determined pericytes as SMA-positive cells adjacent to lumen containing red blood cells. As to human cancers: the leaky phenotype includes ovarian, colon, and intestinal-type gastric cancer, whereas the tight phenotype includes pancreatic and diffuse-type gastric cancer.

Fig. 4. A Schematic Summary of this Work

Tumors can be classified into two categories: those with less coverage of tumor neovasculature by pericytes (left column) and those with more (right column). The former represent a leakier phenotype for nanoDDS, while the latter do not. We have called these “leaky phenotype” (left column) and “tight phenotype” (right column). To view these contrasting phenotypes, HE staining of C26 colon cancer and BxPC3 pancreatic cancer is shown. Vasculature, lumen containing red blood cells in HE staining, is shown with arrowheads. Bars, 100 µm. Inset: a representative image of vasculature at higher magnification. Bars, 10 µm. Scheme: NanoDDS (green particles incorporating red dots in the upper panel) can more easily extravasate through blood vasculature (BV) of the leakier phenotype and accumulate (green area in the lower panel) throughout the tumor tissue, but in the tighter phenotype, more thickly covered by pericytes (green cells attached to BVs in the upper panel, and yellow circles around BVs in the lower panel), nanoDDS extravasates little and does not accumulate sufficiently in target tissue except for areas adjacent to vasculature. As to human cancers: the leaky phenotype includes ovarian, colon, and intestinal-type gastric cancer, whereas the tight phenotype includes pancreatic and diffuse-type gastric cancer.
tumors although associated with the same organ. The results suggest that devastating cancers have more pericyte-coverage than relatively curable cancers. These observations suggest that pericyte-coverage is a factor in determining how difficult cancer is to treat.

To overcome the difficulty of treating pancreatic cancer, we proposed combined use of transforming growth factor-beta (TGF-β) inhibitor with nanoDDS\(^2\); this reduces pericytes and increases accumulation of nanoparticles in stroma rich models. This increased potency and showed accumulation of nanoparticles in the pancreatic cancer xenograft, not only those incorporating anti-cancer agents, but also those incorporating expression vector for green fluorescent protein (GFP)\(^3\) or magnetite particles such as the contrast medium for magnetic resonance imaging (MRI).\(^3\) We are still unsure, however, if this pancreatic cancer model provides a clear enough picture, for clinical use, of pericyte-coverage since it is greater in human samples. In part, this may be due to an impaired immune response in the mice we use for xenograft. Therefore we need, in future, to establish models more relevant to human tumors in terms of their vascular structure in order to investigate effective treatment using nanoDDS.

Cancer models in animals have mainly been established for the purpose of investigating biology of tumor cells, not tumor environment including vasculature. Therefore most available tumor xenografts have tumor cells plus sufficient vasculature to nourish tumor cells.\(^3\)\(^3\)\(^3\) To develop nanoDDS that utilize differences in porosity of vasculature to reduce side effects, we need, therefore, to establish better experimental models clinically relevant in terms of vascular characteristics.

Although anti-angiogenic therapy is now clinically available, most experimental evidence is still based upon xenografts with leaky vasculature.\(^3\)\(^4\)\(^3\)\(^4\) Therefore we need to optimize use of tumor vasculature models based upon the actual histology of human tumors, to establish experimental models of more clinical relevance.

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