Editorial

A complicated interpretation of a therapeutic effect with humanized mice using a novel peptide platform

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Comment on: Karageorgis A, Claron M, Juge R, *et al.* Systemic Delivery of Tumor-Targeted Bax-Derived Membrane-Active Peptides for the Treatment of Melanoma Tumors in a Humanized SCID Mouse Model. Mol Ther 2017;25:534-46.

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In the article "Systemic delivery of tumor-targeted Bax-derived membrane-active peptides for the treatment of melanoma tumors in a humanized SCID mouse model", Karageorgis and colleagues, cyclic RGD conjugated apoptosis-inducing peptide platform strongly inhibited the growth of melanoma tumors in humanized SCID mouse model (1). Karageorgis and colleagues concluded that the robust therapeutic effect was through weak apoptosis and an immunostimulatory effect conferred by their original peptide platform that contained a macrocyclic peptide scaffold RAFT, apoptosis-inducing peptide Poro2 and cyclic RGD peptide (cRGD), whose $\alpha_V\beta_3$ integrin receptor was highly expressed in melanomas (2).

The authors first optimized an apoptosis-inducing peptide, which was a part of the Bax protein. Their results indicated that the peptide was composed of amino acid residues from 109 to 127 of the entire Bax sequence (called Poro2) for inducing the disruption of mitochondria extracted from various melanoma cell lines by measuring the extent of leakage of the mitochondrial heat shock protein 70 (HSP70) and cytochrome C (CytC) as an apoptosis marker. As this apoptosis was suppressed by the addition of Bcl-2 or cholesterol, both of which are known to inhibit pore-formation on mitochondrial membranes, the site-of-action of Poro2 was expected to be pores on mitochondria membraned. Further, to protect Poro2 from being degraded by proteases, they synthesized a Poro2 peptide with D-form amino acids. Poro2 that was made up of D-amino acids was still active on isolated mitochondria.

Since the Poro2 peptide is not very soluble, it was difficult to synthesize the peptide by solid-phase synthesis. Therefore, the method used was optimized for large scale synthesis by introducing the depsipeptide, in which an ester bond is introduced into peptides instead of an amide bond. The depsipeptide was more soluble than the original Poro2 peptide, and was stable at an acidic pH. When the pH was changed from 1.0 to 7.0 after the solid-phase synthesis, a spontaneous O-N acyl shift occurred in the ester bond of depsipeptide, resulting in the formation of the original Poro2 peptide. To deliver the Bax-derived peptide Poro2 to tumor tissue, the Poro2 was linked to a target moiety composed of four cRGD peptides and a macrocyclic peptide scaffold RAFT, which was referred to as RAFT-c(RGD)$_4$-Poro2. The methodology used for the large-scale preparation of the hydrophobic peptide appears to be excellent. In addition, in an *in vitro* study, they showed that RAFT-c(RGD)$_4$-Poro2 induced apoptosis and subsequent cell death, based on MTT assays and measuring the amount of cleaved caspase 3, a marker of apoptosis.

To evaluate the therapeutic effect of their original RAFT-c(RGD)$_4$-Poro2 system, human melanoma Me275-bearing humanized mice were treated with the RAFT-c(RGD)$_4$-Poro2. Since it is difficult to evaluate the impact of immune cells on a therapeutic effect using immunodeficient mice, the authors manipulated the mice so as to contain human immune cells by inoculating HLA-A0201$^+$ and CD34$^+$ hematopoietic progenitor cells into immunodeficient NOD-SCID IL2γRC$^{-/-}$ at 3 weeks before the experiment. The continuous intraperitoneal injection of RAFT-c(RGD)$_4$-Poro2 led to the substantial inhibition of tumor growth

in comparison with free Poro2 or RAD-conjugated Poro2 (a control peptide). Both Poro2 and c(RAD)-conjugated Poro2 exerted no detectable inhibitory effect on tumor growth. Strangely, changes in the numbers of apoptotic cells after the treatment, despite the obvious shrinkage of tumor tissue, were not statistically significant, but were slightly increased.

To elucidate the possible reason why the injection of RAFT-c(RGD)$_4$-Poro2 successfully reduced tumor growth, the authors next examined the angiogenic protein angiogenin and fibroblast growth factor (FGF)-2, and the chemokines monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) and interferon gamma-induced protein 10 (IP10) in plasma from the mice. Among them, the level of FGF-2, which is related with angiogenesis (3), was significantly decreased. On the other hand, MCP1 was up-regulated, which induced the infiltration of monocytes (4). The authors concluded that these changes in chemokine production resulted in tumor growth being inhibited via immune reactions. However, no significant increase in the number of infiltrating immune cells, such as natural killer (NK) cell and T cells, was observed in the tumor tissue. Further, MCP-1 is known as poor prognosis marker, since MCP-1 attracts monocytes, resulting in angiogenesis (5). Although the RAFT-c(RGD)$_4$-Poro2 that they developed was very sophisticated and highly original scientifically, the mechanism responsible for its inhibiting tumor growth in the absence of apoptosis, immune reactions nor anti-angiogenic effects was quite unclear, but very interesting.

One possible mechanism for this anti-cancer effect would be priming tumor microenvironment (TME), including ECM remodeling and vascular normalization. TME is a term for an abnormal condition associated with increases in various cell populations, such as macrophages, fibroblasts and mesenchymal cells, and abundant extracellular matrixes (ECM) including type I collagen and hyaluronan (6). In such a condition, the proliferation of cancer cells is enhanced via the produced chemokines, hypoxia and mechanical stress (7,8). In abnormal TME, vascular permeability is increased and the vasculature is compressed due to solid stress exerted by proliferating cells and ECM (9-11). In this compressed vasculature, the blood flow is stagnant, resulting in tumor tissue hypoxia due to a lack of available oxygen. RAFT-c(RGD)$_4$-Poro2 induced the production of apoptosis proliferative cancer cells, and consequently mechanical stress exerted by the vasculature would be relieved. Actually, killing cancer cells by radiation therapy decreases interstitial fluid pressure, and consequently increased the partial pressure of oxygen (12). Accordingly, appropriate oxygenation from the decompressed vasculature was altered in hypoxic tumor tissue compared to the normoxic type by apoptosis resulting from the RAFT-c(RGD)$_4$-Poro2. Since improving hypoxic tumor tissue is known to contribute to a robust immune therapeutic effect (13), such a TME alteration by the injection of RAFT-c(RGD)$_4$-Poro2 would contribute to tumor inhibition.

Moreover, as the receptor of cRGD, $\alpha_V\beta_3$ integrin is also expressed not only in melanoma cells but also in tumor endothelial cells (TECs), and RAFT-c(RGD)$_4$-Poro2 could also induce apoptosis in abnormally rapid growing TEC cells. Actually, the angiogenic chemokine FGF-2 was suppressed as the result of the injection of the RAFT-c(RGD)$_4$-Poro2, the levels of CD31$^+$ cells (an endothelial cell marker) were slightly diminished, even if not statistically different. This reduction in the number of TECs could lead to vascular normalization because it was reported that inducing apoptosis in endothelial cells resulted in vascular normalization (14). A previous report revealed that, when an antibody against vascular endothelial cell growth factor receptor 2 (VEGFR2) DC101, a treatment that improved hypoxic tumors via vascular normalization, the macrophage subtype was changed from an immunosuppressive M2-type to an immunostimulatory M1-type (15). M1-type macrophages support the killing of immune cells. Taken together, in the article on RAFT-c(RGD)$_4$-Poro2, altering the macrophage type through oxygenation via vascular normalization might support the immune activation by transplanted human hematopoietic cells.

The findings reported in this manuscript suggest that therapeutic outcomes with nano-carriers are strongly influenced by TME. However, the strategy for controlling TME might be dependent on the type of cancer cells. We previously reported that the delivery of siRNA against VEGFR2 improved hypoxia in tumor tissue, and thus M1-type macrophages infiltrated into tumor tissue and degraded ECM by a matrix metalloproteinase in a human renal cell carcinoma OS-RC-2-bearing model (16,17). The ECM degradation resulting from vascular normalization enhanced the intratumoral penetration of nanoparticles and synergistically enhanced the action of doxorubicin-loaded liposomes. On the other hand, this improvement in intratumoral distribution of a nano-carrier by vascular normalization was not observed in colorectal cancer HCT116-bearing mice (18). These results suggest that the type of cancer needs to be taken into consideration in the

appropriate optimization of TME. It was also reported that the innate immune response via the toll-like receptor varied, even among the same varieties of lung cancer (19). Thus, the issue regarding the response for controlling TME and therapeutic efficacy against immunotherapy or/and nano-carrier varies among cancer types is currently veiled (20). This difference prevents us from predicting an appropriate nano-carrier, and how we should regulate TME for suitable cancer treatment by a nano-carrier prior to the treatment.

We currently infer that tumor stromal morphology has a substantial effect on the therapeutic outcome of a nano-carrier in TME based on a report by Smith *et al.* that vessels surrounding cancer cells (tumor vessel; TV) or vessels surrounding stromal cells (stroma vessel; SV) define the VEGF blockade-mediated therapeutic effect (21). In fact, siRNA against VEGFR2 was reported to improve the intratumoral distribution of nanoparticles in TV-type renal cell carcinoma, whereas in SV-type colorectal cancer as mentioned above in our manuscript. SV-type cancer was basically difficult to treat with a nano-carrier due to the fact that the extravasation and distribution of nano-particles is inhibited by presence of abundant ECM and stroma cells (22). Much worse, cancer types that affect many patients (lung cancer, colon cancer, prostate cancer) are SV-type cancers. In these fibrotic SV-type cancers, reducing the level of stromal cells would be effective. For example, Kano *et al.* reported that the inhibition of transforming growth factor (TGF)-β, which was a chemokine for migration and the proliferation of stromal cells, significantly improved the penetration of nano-carriers in one of most fibrotic model of pancreatic cancer (23). In summary, it should be important to systemize and classify differences in TME among cancer types in order to properly treat each cancers.

Another serious issue is the differences between preclinical and clinical cancer. ECM in clinical cancer is likely to be more abundant than that in a preclinical cancer model (24). This difference between human and mouse models made it difficult to understand the exact details of TME in humans. Additionally, to inoculate human cancer cells, it is necessary to use T cell-deficient mice (nude mice) or T cells and B cell-deficient mice (severe combined immunodeficiency mice), which can obscure experimental results, specifically involved with immune cells. Considering these aspects, the manuscript reported by Karageorgis and colleagues would be extremely valuable for evaluating TME reflecting clinical specimens. In the future, a refined experimental cancer model which precisely imitates stromal TME in clinical cancer should be developed for evaluating the behavior of nano-carriers in human TME and understanding specifically nano-carriers function.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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