Selective targeting of interferon gamma to stromal fibroblasts and pericytes as a novel therapeutic approach to inhibit angiogenesis and tumor growth

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Conflict of Interest: Klaas Poelstra is a co-inventor of pPB-HSA patent and is a co-founder and CSO of Biorion Technologies, Netherlands and holds <5% stocks in the company. Jai Prakash is VP Preclinical, Biorion Technologies and acts as an advisory member.

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ABSTRACT

New approaches to block the function of tumor stromal cells such as cancer-associated fibroblasts and pericytes is an emerging field in cancer therapeutics as these cells play a crucial role in promoting angiogenesis and tumor growth via paracrine signals. Because of immunomodulatory and other antitumor activities, interferon-gamma (IFNγ), a pleiotropic cytokine, has been used as an anti-cancer agent in clinical trials. Unfortunately only modest beneficial effects, but severe side effects, were seen. In this study, we delivered IFNγ to stromal fibroblasts and pericytes, considering its direct anti-fibrotic activity, using our Platelet-Derived Growth Factor-beta Receptor (PDGFβR)-binding carrier (pPB-HSA), as these cells abundantly express PDGFβR. We chemically conjugated IFNγ to pPB-HSA using a heterobifunctional PEG linker. In vitro in NIH3T3 fibroblasts, pPB-HSA-IFNγ conjugate activated IFNγ-signaling (pSTAT1α) and inhibited their activation and migration. Furthermore, pPB-HSA-IFNγ inhibited fibroblasts-induced tube formation of H5V endothelial cells. In vivo in B16 tumor-bearing mice, pPB-HSA-IFNγ rapidly accumulated in tumor stroma and pericytes and significantly inhibited the tumor growth while untargeted IFNγ and pPB-HSA carrier were ineffective. These antitumor effects of pPB-HSA-IFNγ were attributed to the inhibition of tumor vascularization, shown with α-SMA and CD-31 staining, respectively. Moreover, pPB-HSA-IFNγ induced MHC-II expression specifically in tumors compared to untargeted IFNγ, indicating the specificity of this approach. This study thus shows the impact of drug targeting to tumor stromal cells in cancer therapy as well as provides new opportunities to utilize cytokines for therapeutic application.
INTRODUCTION

In the past decade, the complexity of the tumor microenvironment has been extensively studied, and this knowledge has contributed to the development of new therapies for cancer (1). Apart from cancer cells, solid tumors contain large amounts of tumor stroma comprising a variety of cell types such as cancer-associated fibroblasts (CAFs), pericytes, endothelial cells, infiltrated immune cells, and cancer stem cells. Among them, CAFs are the major cell type that play a crucial role in tumorigenesis and metastasis (1, 2) by secreting various cytokines and growth factors (e.g. VEGF, HGF, SDF-1α), which act in a paracrine/exocrine fashion on other cell types, thereby activating tumor-inducing processes (2-4). In addition to CAFs, pericytes are another important cell type, having phenotypic characteristics of mesenchymal cells and fibroblasts. These pericytes stabilize endothelium by surrounding the blood vessels and support angiogenesis by secreting VEGF (1). Both stromal fibroblasts and pericytes, collectively referred here as stromal cells, express high levels of platelet-derived growth factor-beta receptor (PDGFβR) and its expression in tumor stroma has been inversely correlated with the survival rate in patients with different types of cancer (5, 6). Also, studies have shown that inhibition of the functions of these stromal cells using a PDGFβR inhibitor (imatinib) leads to inhibition of angiogenesis and thereby reduction in tumor growth (7, 8). These data indicate the key role of the tumor stromal cells in tumor development; therefore selective targeting to stromal cells for cancer therapeutics is of great interest and could provide highly attractive strategies to treat cancer.

Among potent anticancer agents, interferon gamma (IFNγ) has been shown to possess multiple potent anti-tumor properties. IFNγ is an immunomodulatory cytokine produced by immune cells (mainly NK cells and subsets of T cells) and is physiologically involved in promoting innate and adaptive immune responses (9). It interacts with the IFNγ receptor and activates the JAK-STAT1 signaling pathway, which regulates transcription of various genes. IFNγ, apart from its physiological functions, has been extensively explored as a therapeutic cytokine for various diseases such as immunodeficiency diseases, chronic inflammatory diseases, fibrosis, tumors and atypical mycobacterial infections in pre-(clinical) studies (10-12). However, most clinical trials failed (13-16) and its clinical application is limited due to its side effects on
non-target cells as IFNγ receptors are present on almost all cell types. The antitumor response of IFNγ has been shown to be mainly associated with its immunological effects, but also non-immunological effects such as direct killing of tumor cells and inhibition of proliferation of endothelial cells have been proposed. In addition, IFNγ has been shown to display strong anti-fibrotic effects in different fibrosis models in lung, liver and kidneys (17-20) by inhibiting activation and proliferation of fibroblasts.

Since stromal cells highly contribute to angiogenesis and tumor growth, we hypothesized that interference in the tumor-promoting activities of these cells by the local delivery of IFNγ might inhibit the tumor growth. We have designed a PDGFβR-recognizing drug carrier (pPB-HSA) comprised of PDGFβR-binding cyclic peptides (pPB) conjugated to human serum albumin (HSA) (21, 22) for specific targeting to PDGFβR-expressing tumor stromal cells. Furthermore, we have shown that pPB-mediated targeting of IFNγ to hepatic stellate cells, expressing high levels of PDGFβR during liver fibrosis, completely abolished advanced liver cirrhosis in mice (23). In the present study, we delivered IFNγ to stromal fibroblasts and pericytes using pPB-HSA carrier to impair angiogenesis thereby inhibiting the tumor growth, while avoiding IFNγ-mediated off-target effects. To effectuate this, we conjugated IFNγ to pPB-HSA and examined the synthesized conjugate for its therapeutic efficacy in vitro and in vivo.
MATERIALS AND METHODS

Cell lines

Murine NIH3T3 fibroblasts, B16-F10 melanoma cells, and RAW264.7 macrophages were obtained from American type culture collection (ATCC, Rockville, MD). H5V heart capillary endothelial cell line was kindly provided by Dr. Vecchi (Mario Negri, Institute for Pharmacological Research, Milan, Italy) to UMCG Groningen. RAW264.7, NIH3T3, H5V and B16 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% FBS and antibiotics. No authentication for cell lines was performed by the authors.

Synthesis and characterization of pPB-HSA-IFNγ conjugate

The synthesis procedure of pPB-HSA-IFNγ conjugate has been described earlier (24). The brief methodology has been provided in the supplementary methods. The pPB-HSA-IFNγ conjugate was characterized using Western blot analyses and the biological activity was assessed with a nitric oxide (NO) release assay in RAW cells as described earlier (24).

In vitro binding of the IFNγ conjugate to mouse 3T3 fibroblasts

Cells were cultured overnight in Lab-Tek (Nunc, Roskilde, Denmark) and incubated with pPB-HSA-IFNγ (1μg/ml) for 2h. To block the PDGFB-R-mediated binding, anti-PDGFB-R IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was added 1h before adding IFNγ conjugate. Then, cells were stained with anti-pPB antibody.

In vitro effects of the IFNγ conjugate in mouse 3T3 fibroblasts

Cells (3x10^4 cells/24-well and 7.5x10^4 cells/12-well plate) were cultured for overnight and starved with 0.5% FCS containing medium for 24h. Cells were then incubated with 5ng/ml human recombinant TGFβ1 (Roche, Mannheim, Germany) with or without IFNγ (16nM), pPB-HSA-IFNγ (equivalent to 16nM IFNγ) and pPB-HSA (molar equivalent) for 48h. Subsequently, cells were stained for collagen-I or α-SMA and were analyzed for gene expression (supplementary materials and methods).
The IFNγ signaling p-STAT1α was analyzed using Western blot analysis in 3T3 fibroblasts, 24h after incubating with different compounds as mentioned above. Western blot was performed from the cell lysates using rabbit monoclonal anti-pSTAT1α antibody (1:1000, Cell signaling technology Inc., Beverly, MA) and β-actin (1:5000, Sigma) as detailed in supplementary materials and methods.

Wound-healing assay

NIH3T3 cells were grown for 24h and starved overnight in 0.5% FCS containing medium. A standardized scratch was made using a 200μl pipette tip fixed in a holder. Then, cells were incubated with IFNγ (16nM), pPB-HSA-IFNγ (equivalent to 16nM IFNγ) or pPB-HSA (molar equivalent). Digital pictures of wounds were captured at t=0h and t=24h and were analyzed by NIH-ImageJ software to calculate the area of the scratch wound and represented as the percentage of wound healed relative to the controls.

To study the indirect effect of fibroblasts (3T3) on tumor cells (B16), 3T3 cells (1x10^5) were grown for 24h, starved for overnight and then incubated with TGFβ (5ng/ml) with or without IFNγ (16nM), pPB-HSA-IFNγ (equivalent to 16nM IFNγ) or pPB-HSA for 24h. Thereafter, cells were washed thrice and incubated with fresh starved medium for 24h. This conditioned medium was put on B16 cells (5x10^4, cultured for 48h) to perform the wound-healing assay as described above.

In vitro matrigel tube-formation assay

The fibroblast-mediated paracrine effects of IFNγ and pPB-HSA-IFNγ conjugate on endothelial cells (H5V) were examined using the matrigel tube-formation assay (25). Briefly, 3T3-conditioned medium collected after different treatments as mentioned above was added to HSV cells (4x10^4) plated on the matrigel-coated 8-chamber slides (Lab-Tek™). VEGF (10ng/ml, Peprotech) was used a positive control and added directly to H5V cells. After 20h incubation, tubes were visualized, counted and represented as relative percentage of tube formation.

Subcutaneous B16 tumor mouse model

All animals (male C57BL/6 mice, 20-22g, Harlan, Ziest, Netherlands) received ad libitum normal diet and 12h light and 12h dark cycle. Experimental protocols were approved by the Animal Ethics Committee.
Subcutaneous tumors were induced by injecting B16 cells \((1 \times 10^6 \text{cells/}100 \mu\text{l PBS/mouse})\) in the left flank. Tumor size was measured using a digital vernier Caliper and tumor volume was established using the following formula \((a \times b^2/2)\) where “a” and “b” denotes the length and width of a tumor, respectively.

To determine the therapeutic efficacy, on day 5 after tumor cell injection (when tumors were formed) mice were randomized into 4 groups and were injected intravenously with 5μg IFNγ/mouse/day (n=5), pPB-HSA-IFNγ (equivalent to 5μg IFNγ, n=5), pPB-HSA (molar equivalent to pPB-HSA-IFNγ, n=4) or vehicle (PBS, n=5) on days 5, 7, 9, 11, 13, 15 and 17. IFNγ amount in the conjugate was analyzed by Western blot. The IFNγ dose was based on our previous studies in liver fibrosis and literature (26, 27). Animals were sacrificed and blood, tumors and other organs were collected for further analysis. To examine the IFNγR signaling \((\text{pSTAT1α})\) \textit{in vivo}, 20μg of protein from tumor lysates were analyzed by Western blot using anti-pSTAT1α, STAT1α and β-actin antibodies.

For the bio-distribution of pPB-HSA-IFNγ in B16 tumor-bearing mice with tumor size of approximately 2000mm³, a single dose (5μg/mouse) of pPB-HSA-IFNγ was injected intravenously 15min prior to sacrifice. Cryosections from tumors and other tissues were stained with anti-pPB IgG for \textit{in vivo} localization.

**Immunohistochemistry and immunofluorescence**

Cryosections (4μm) of tumors and organs were cut and the staining protocol was followed as described earlier (23). Antibodies with their dilution and the detailed method have been described in supplementary methods.

**Statistical analyses**

Data are presented as the mean ± standard error (SEM). Multiple comparisons between different groups were performed by one-way ANOVA with Bonferroni post-test unless otherwise mentioned in the figure legends.
RESULTS

Expression of IFNγR-II and PDGFβR receptors in subcutaneous B16 tumors and other tissues in mice

We initially compared IFNγR-II and PDGFβR expression in B16 tumors and other organs and found that both receptors were highly expressed in tumor stroma (Supplementary Fig. S1). In other organs, IFNγR-II was also strongly expressed but PDGFβR expression was low as compared to tumors. In addition, many immune cells especially macrophages strongly express IFNγR-II (28). Systemic administration of IFNγ will therefore elicit effects in multiple cells in many different organs and immune cells, and distribution to tumors will be relatively low. The high PDGFβR expression on tumor stromal cells in tumors relative to all other tissues supports our notion for the suitability of this receptor for cell-selective targeting of IFNγ.

Characterization of pPB-HSA-IFNγ conjugate

Western Blot analysis of the synthesized PDGFβR-targeted IFNγ conjugate (see diagram in Fig. 1A) using anti-HSA and anti-IFNγ antibodies showed coupling of about two IFNγ per pPB-HSA molecule. Nitric oxide release assay in murine RAW264.7 monocytes showed that there was no loss of biological activity of pPB-HSA-IFNγ as also shown earlier (24). A clear binding of pPB-HSA-IFNγ to 3T3 cells was observed, which was strongly inhibited by anti-PDGFβR IgG, demonstrated its PDGFβR-related specificity (Fig. 1B). Upregulation of PDGFβR expression on 3T3 fibroblasts after activation with TGFβ (Fig. 1C) favors the binding of the construct to the activated fibroblasts and pericytes, known to express high PDGFβR (2, 4).

Modification of IFNγ might cause a loss of activity, however activation of pSTAT1α signaling and MHC-II expression in 3T3 cells by pPB-HSA-IFNγ clearly indicate a full retention of the IFNγ-related activity after chemical modification (Fig. 1D).

pPB-HSA-IFNγ inhibits fibroblasts activation

Furthermore, we investigated the inhibitory effects of IFNγ conjugate on fibroblast activation. Both IFNγ and pPB-HSA-IFNγ substantially inhibited TGFβ-induced activation of 3T3 fibroblasts as demonstrated by protein and gene expression of α-SMA (Fig. 2A and 2B). Additionally, they inhibited TGFβ-induced
protein and gene expression of collagen-I and fibronectin-I (p<0.01) (Supplementary Fig. S2A-C). In contrast, no inhibitory effects of pPB-HSA rules out the possibility of PDGFβR-blocking effects. Furthermore, IFNγ or pPB-HSA-IFNγ significantly inhibited the migration of fibroblasts, shown with wound-healing assay (Fig. 2D). Earlier, we have shown that targeted IFNγ inhibits the PDGF-BB-induced proliferation of fibroblasts (24). In our wound-healing assay, however, absence of apoptosis and TGFβ-related proliferation stresses that inhibition of wound healing was mainly caused by inhibition of cell migration (Supplementary Fig. S2C and S2D). These results demonstrate that both IFNγ and PDGFβR-targeted IFNγ can block the activation and migration of fibroblasts.

**pPB-HSA-IFNγ inhibits fibroblast-mediated activation of endothelial cells**

Tumor-associated stromal fibroblasts and pericytes activate endothelial cells in a paracrine manner by secreting cytokines and thereby induce angiogenesis (1). In our fibroblasts-induced angiogenesis in vitro model, we found that the conditioned media derived from TGFβ-stimulated fibroblasts but after removal of stimuli enhanced tube formation compared to that of unstimulated media (Fig. 3A and 3B), which was similar to that achieved with VEGF, an endogenous angiogenesis-inducing growth factor. Interestingly, conditioned media derived from 3T3 cells treated with IFNγ or pPB-HSA-IFNγ significantly diminished the TGFβ-induced tube formation capability of fibroblasts (p<0.01, Fig. 3). Of note since the conditioned media lacked all the added stimuli, no direct effect of IFNγ or its construct on endothelial cells was exhibited. Since TGFβ did not cause any proliferative effect on fibroblasts and also the treatments did not cause any changes in proliferation and apoptosis of fibroblasts (Supplementary Fig. S2), the paracrine effects of 3T3 cells were only dependent on the change in the activation state of the cells. These data indicate that selective inhibition of fibroblasts activation with our targeted IFNγ construct may inhibit endothelial cells activation and thereby angiogenesis.

**pPB-HSA-IFNγ specifically accumulates in stromal fibroblasts and pericytes in vivo**

To demonstrate the tumor stroma targeting in vivo, we investigated the accumulation of pPB-HSA-IFNγ in tumors and various organs, 15min after intravenous injections. Using anti-pPB immunostaining, we
found that pPB-HSA-IFN\(\gamma\) rapidly accumulated in tumors especially in tumor stroma (Fig. 4A) where PDGF\(\beta\)R was highly expressed (see Supplementary Fig. S1). pPB-HSA-IFN\(\gamma\) was also found in livers where pPB staining was localized in the sinusoidal lumina. In other organs such as kidneys, heart and lungs, there was almost no staining detectable (Fig. 4A), which correlates with the low PDGF\(\beta\)R expression in these organs (see Supplementary Fig. S1). Since pericytes surrounding tumor endothelium express high PDGF\(\beta\)R, we performed co-immunostaining for PDGF\(\beta\)R and pPB-HSA-IFN\(\gamma\) (anti-pPB), and found a co-localization of the conjugate with pericytes (Fig. 4B). These results demonstrate that pPB-HSA-IFN\(\gamma\) conjugate specifically accumulates into PDGF\(\beta\)R-expressing tumor stromal fibroblasts and pericytes.

**pPB-HSA-IFN\(\gamma\) reduces tumor growth in vivo by inhibition of angiogenesis**

In B16-F10 subcutaneous tumor-bearing mice, treatment with pPB-HSA-IFN\(\gamma\) significantly reduced the progression of this malignant tumor (Fig. 4C) while PBS, IFN\(\gamma\) or pPB-HSA did not inhibit it. The enhanced anti-tumor effect of the targeted construct was attributed to an increased tumor uptake of pPB-HSA-IFN\(\gamma\) as also demonstrated by the activation of the IFN\(\gamma\) signaling (pSTAT1\(\alpha\)) in tumors from pPB-HSA-IFN\(\gamma\)-treated animals (p<0.05 vs. PBS) as compared to other treatment groups (Fig. 5A).

We further explored the effect of the targeted construct on stromal cells and found that \(\alpha\)-SMA-positive cells (fibroblasts and pericytes) were markedly less prevalent in targeted IFN\(\gamma\)-treated tumors compared to control tumors (Fig. 5B). Also, there was a significant reduction (p<0.01) in the pericyte population in pPB-HSA-IFN\(\gamma\)-treated mice, shown with reduction of \(\alpha\)-SMA staining around the blood vessels (Fig. 5B). In line with our *in vitro* tube formation assays, we found a significant reduction (p<0.01 vs. IFN\(\gamma\) or pPB-HSA) in angiogenesis with the construct, as shown with the quantitative analysis of CD31-stained lumen area of tumor blood vessels (Fig. 5C). In addition, we performed cleaved caspase-3 staining in tumors and found that neither free IFN\(\gamma\) nor pPB-HSA-IFN\(\gamma\) induced apoptosis (data not shown), excluding a possibility of direct pro-apoptotic effect of the compounds on tumors. Since IFN\(\gamma\) is a proinflammatory cytokine and tumor inhibitory effects of the conjugate could be immune-mediated, we performed CD68 (a common marker for monocytes, macrophages, kupffer cells, dendritic cells), CD4 and CD8 (markers for T-lymphocytes) stainings on the tumor tissues and found no significant differences among...
different treatment groups (Supplementary Fig. S3). These data demonstrate that the most of beneficial effects of targeted IFNγ are attributed to the direct inhibition of stromal fibroblasts- and pericyte-supported blood vessel formation.

To examine the effect of targeted IFNγ on other organs, we performed MHC-II immunostaining in tumors and liver, lungs, and kidneys and performed semi-quantitative analyses. We found that targeted IFNγ induced MHC-II expression significantly more in tumors compared to other treatments (Fig. 6). In other organs, there was no significant increase with any of the treatments. In the biodistribution study, we observed the distribution of the conjugate in liver sinusoids, but absence of liver inflammation (detected by CD68 immunostaining; Supplementary Fig. S3) in the conjugate-treated livers rules out the possibility of side effects in liver. Furthermore, we examined the body weight and blood parameters in all groups and found no adverse effects of the treatments (Supplementary Table S1 and Supplementary Fig. S4).

Taken together, these results demonstrate that selective targeting of IFNγ to tumor stroma inhibits tumor growth indirectly by inhibition of angiogenesis. The targeted construct displayed significantly more potent antitumor activity than native IFNγ, with no significant side effects in other organs.
DISCUSSION

The present study reveals that specific targeting of IFNγ to stromal fibroblasts and pericytes through a PDGFβ receptor-recognizing carrier leads to inactivation of these key cell types in tumors and thereby reduces the tumor growth in vivo. Epithelial-derived tumors are generally characterized by the generation of mesenchymal-derived stromal cells, including intratumoral and peritumoral fibroblasts and tumor vasculature-associated pericytes. The paracrine signals induced by these cells have been implicated in tumor growth, angiogenesis, invasion and metastasis (2, 3). Selective targeting of anti-fibrotic compounds to these cells, as shown with IFNγ in the present study, may therefore pose a novel approach for the development of a new potential anti-cancer therapy.

Cell-specific targeting to stromal cells is an unexplored area of research and so far only small molecule inhibitors of Hedgehog, Fibroblasts Activation Protein (FAP) and PDGFR have been used to demonstrate the anti-stromal effects on tumor growth (8, 29, 30) and drug uptake (7, 31). Until now, IFNγ has been shown to possess no/moderate anticancer activity in experimental models (27). In fibrosis field, IFNγ has been well explored as an anti-fibrotic cytokine due to its direct effects on fibroblasts, and examined in clinical studies for idiopathic pulmonary fibrosis and liver fibrosis, though remained ineffective (13, 14). Main reasons for its clinical failure are its poor pharmacokinetics and severe side effects. IFNγR is highly expressed on immune cells (28) and numerous other cells in different organs, as shown in Supplementary Fig. S1, which leads to severe systemic adverse effects. Therefore, targeted delivery of IFNγ to specific key disease-inducing cells is prerequisite to enhance its therapeutic efficacy and to reduce its side effects.

Many attempts have been made to deliver IFNγ to tumors using liposomes, polymer gels, microspheres and nanoparticles (32-34). In these approaches, however, cell-selective targeting is lacking which might result in systemic side effects in long-term treatment. Delivery of IFNγ specifically to tumor blood vessels using a GCNGRC peptide (NGR) has also been attempted to induce immune-mediated antitumor effects (27). However, IFNγ-NGR construct induced potent anti-tumor effects at very low doses (0.005μg/kg), while non-targeted IFNγ induced little or no effect at the dose of 0.003-250μg/kg. At higher
doses of both untargeted and targeted IFNγ were ineffective due to induction of immune-mediated counter-regulatory mechanisms (27), and moreover, multiple treatments at low doses induced resistance to the therapy (35). In the present study, however, we applied a different approach and targeted IFNγ to both stromal fibroblasts and pericytes using a PDGFβR-targeting peptide. This strategy has many advantages over other approaches because 1) stromal fibroblasts and pericytes compose the largest component in a tumor providing a large area for targeting; 2) these cells strongly participate in many tumor-promoting processes, inhibition of which may lead to hampering of tumor growth; 3) PDGFβR expression is highly expressed on these stromal cells compared to tumor cells and normal tissues; 4) stromal cells are likely to be more genetically stable and commonly present in multiple tumor types; 5) furthermore, the antitumor effects are mostly exhibited through its anti-fibrotic activity than immunomodulatory effects, and therefore chances of counter-regulatory mechanisms, as exemplified above, would be minimal.

Both CAFs and pericytes are mesenchymal cell types and commonly express PDGFβR and α-SMA whereas pericytes present in normal tissues do not express α-SMA (2, 4). TGFβ-activated 3T3 fibroblasts, as shown in this study, had also high expression of these markers, depicting the characteristics of stromal fibroblasts. Inhibition of activation and migration of these cells as well as decrease in the production of extracellular matrix by IFNγ and pPB-HSA-IFNγ indicates the potent anti-fibrotic effects of these compounds. As expected, free and targeted IFNγ showed similar effects in vitro because of no constrains for binding to IFNγR. The real impact of stromal cells in a tumor is exerted by their strong paracrine actions through which they induce angiogenesis, invasion, metastasis and tumorigenesis (2, 4). Through the paracrine mimicking in vitro experiments, we demonstrated that treatment of fibroblasts with targeted IFNγ strongly inhibited the fibroblasts-induced tube formation of endothelial cells. These data support the notion that the selective inhibition of stromal cells in vivo may inhibit their paracrine action and thereby the tumor growth.

Cell-selective targeting in vivo is a challenging task mainly due to non-specificity of a target receptor. In our approach, targeting to stromal cells through PDGFβR caused a rapid accumulation of pPB-
HSA-IFNγ in tumor stroma and pericytes in subcutaneous tumors. Although PDGFβR is known to be expressed on many cell types in different organs, as a matter of fact its expression is mainly high during early developmental stages but quite low in normal tissues (36, 37). In many pathological conditions, PDGFβR expression increases remarkably, especially in fibrotic diseases and in tumor stroma (2, 38). For the same reason, we found a negligible distribution of pPB-HSA-IFNγ in normal organs except in liver sinusoids, which is most likely due to its presence in circulation. We have shown in an earlier study that a pPB-HSA-doxorubicin conjugate was visible in liver sinusoids after 30min of an intravenous injection but disappeared after 2h (22). Moreover, in the present study no significant induction in MHC-II and CD68 expression in livers with the conjugate clearly indicates no side-effect in liver. These data further signifies the tumor specificity of the therapy.

The potential benefits of the targeted approach were observed *in vivo* where targeted IFNγ significantly reduced the tumor growth while untargeted IFNγ (at the equivalent dose) was ineffective. A substantial induction of pSTAT1α expression in tumors by pPB-HSA-IFNγ confirmed its IFNγ-mediated local effects. In contrast, free IFNγ or the carrier did not significantly enhance the pSTAT1α in tumors. Reduction in α-SMA expression in the tumor-associated fibrous tissue and around blood vessels with targeted IFNγ clearly demonstrated the deactivation and/or reduction of fibroblasts and pericytes, which resulted in the antitumor effects. In addition, no increase in tumor macrophage or lymphocytes infiltration in the conjugate-treated animals further supports the direct effect on the targeted cells. Since pericytes are directly involved in blood vessel maturation, contribution of pericyte inhibition for antitumor effects is more evident than that of fibroblast inhibition. However, it is difficult to delineate the role of different cell types for these effects.

Induction of systemic side effects by IFNγ has been a major reason for the failure in clinical trials (11). However, at the used doses we did not see any side effect of free IFNγ on body weight and hematological parameters. Also in MHC-II staining analysis, untargeted IFNγ did not induce its expression in tumors and other organs while the conjugate induced it only in tumors (see Fig. 6). Since IFNγ itself did not show side effects at the injected doses, no further improvements were expected from targeted IFNγ.
In conclusion, the present study reveals a novel approach to deliver IFNγ to stromal fibroblasts and pericytes using our PDGFβR-targeting carrier. Blockade of the activation of these cells by targeted IFNγ construct leads to a reduction in tumor growth. These data may form a strong base to develop a novel therapeutic compound for the treatment of cancer as well as provide new opportunities to use cytokines as therapeutic compounds.
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REFERENCES


FIGURE LEGENDS

**Figure 1: Structure and in vitro characterization of pPB-HSA-IFNγ conjugate.** (A) A diagrammatic structure of pPB-HSA-IFNγ conjugate. (B) Fluorescent photographs showing binding of pPB-HSA-IFNγ to mouse 3T3 fibroblasts using anti-pPB immunostaining, which was blocked by anti-PDGFβR antibody. Scale bar, 50μm. (C) Representative bands from the Western blot showing increased PDGFβR expression in TGFβ-activated 3T3 fibroblasts. (D) Western Blot analyses of pSTAT1α and qPCR analysis of MHC-II in 3T3 cells after the treatment with TGFβ (5ng/ml) with or without IFNγ (16nM), pPB-HSA-IFNγ (equivalent to 16nM IFNγ) or pPB-HSA alone. All samples were blotted at the same time and blots were analyzed with the same exposure time. Mean + SEM, n=3, **p<0.01.

**Figure 2: In vitro inhibitory effects of pPB-HSA-IFNγ in mouse 3T3 fibroblasts.** (A) Representative microphotographs showing α-SMA and fibronectin-I staining in 3T3 fibroblasts, incubated with TGFβ (5ng/ml) with or without IFNγ (16nM), pPB-HSA-IFNγ (equivalent to 16nM IFNγ) or pPB-HSA alone. Scale bars, 100μm. QPCR analysis of α-SMA (B) and fibronectin-1 (C) in 3T3 fibroblasts. (D) Representative microscopic images and analysis of wound-healing assay in 3T3 fibroblasts 24h after the incubation with IFNγ (16nM), pPB-HSA-IFNγ (equivalent to 16nM IFNγ) or pPB-HSA (equivalent). Mean + SEM, n=3, *p<0.05 and **p<0.01.

**Figure 3: In vitro inhibition of paracrine effect of activated fibroblasts on endothelial cells.** (A) Representative pictures of endothelial cell tube formation after incubation with conditioned medium from 3T3 cells that were treated with medium alone (control), TGFβ (5ng/ml) with or without IFNγ (16nM), pPB-HSA-IFNγ (equivalent to 16nM IFNγ) or pPB-HSA. Magnification, 40x. (B) Tubes were counted 24h following incubations. VEGF (10ng/ml) was used directly on H5V cells as a positive control. Mean + SEM, n=3, *p<0.05 and **p<0.01.
Figure 4: *In vivo* distribution and the therapeutic effects of pPB-HSA-IFNγ in subcutaneous B16 tumor. (A) The anti-pPB immunostaining illustrates the distribution of the conjugate in tumors and different organs 15min after the intravenous administration of pPB-HSA-IFNγ in B16-tumor bearing mice. Scale bar, 200μm. (B) Representative immunofluorescent photographs depicting specific accumulation of pPB-HSA-IFNγ in pericytes. Anti-pPB (green); anti-PDGFβR (red) and nuclei counterstained with DAPI (blue). Magnification, 400x (C) Tumor growth curve of B16 tumors following intravenous treatment with PBS (n=5), IFNγ (n=5), pPB-HSA-IFNγ (n=5) and pPB-HSA (n=4). *p< 0.05 vs. PBS; §p<0.05 and §§p<0.01 vs. IFNγ; #p<0.05 vs. pPB-HSA; unpaired student’s t-test. Representative pictures of the isolated tumors at the end of the experiment.

Figure 5: *In vivo* effect of pPB-HSA-IFNγ on stromal fibroblasts and pericytes in subcutaneous B16 tumor-bearing mice. (A) Western blot analyses of pSTAT-1α in tumors for pSTAT-1α and STAT1α. The pSTAT1α and STAT1α bands were quantified and neutralized by their respective β-actin controls and then the ratio of pSTAT1α and STAT1α was calculated. All the electrophoresed samples were blotted at the same time and blots were analyzed with the same exposure time. N=3 mice per group. *p<0.05. (B) Representative pictures showing immunostaining for αSMA, a marker for fibroblasts and pericytes, in stromal fibrous capsule (S), tumor (T) and around blood vessels. Scale bar, 200μm. Quantitative analyses of α-SMA immunostaining in tumors using image analysis software. (C) Bar graph showing the lumen area of tumor blood vessels analyzed after CD31 immunostaining on tumor sections. Unpaired student’s t-test, *p<0.05, **p<0.01.

Figure 6: *In vivo* expression of MHC-II in tumors and different organs in B16 tumor-bearing mice. (A) Representative microscopic pictures of MHC-II staining in tumors and different organs. Magnification 200x except livers (100x). (B) Semi-quantitative analyses of MHC-II staining showing the average score (+SEM) in tumors and other organs. n=4-5 mice/group for tumors and n≥3 mice/group for other organs. Unpaired student’s t-test, **p<0.01 vs. PBS, IFNy and pPB-HSA groups. The whole section was scored using the following scoring criteria. Negative section (score 0.5), occasionally positive cells (score 1), a significant
number of positive cells with some negative area (score 2) and areas with strong positive cells but still negative areas (score 3). No tumor or any other organ was completely positive.
Figure 1

**A**

- IFN
- PDGFβR-binding peptide
- HSA
- PEG

**B**

- anti-PDGFβR + pPB-HSA-IFN
- pPB-HSA-IFN
- control

**C**

- PDGFβR
- β-actin

**D**

- pSTAT-1α
- β-actin

(normalized with GAPDH)

TGFβ

(untreated with MHC-I)

1000
500
300
200
100
0

TGFB

(unreleased)

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