A YKL-40 neutralizing antibody blocks tumor angiogenesis and progression: a potential therapeutic agent in cancers

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Abbreviation: mAY, monoclonal anti-YKL-40 antibody; rAY, polyclonal anti-YKL-40 antibody; Flk-1/KDR, VEGF receptor 2, MAPK, mitogen-activated protein kinase; HMVECs, human microvascular endothelial cells; siRNA, small-interfering RNA.

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Abstract: Accumulating evidence has indicated that expression levels of YKL-40, a secreted glycoprotein, were elevated in multiple advanced human cancers. Recently, we have identified an angiogenic role of YKL-40 in cancer development. However, blockade of the function of YKL-40, which implicates therapeutic value, has not been explored yet. Our current study sought to establish a monoclonal anti-YKL-40 antibody as a neutralizing antibody for the purpose of blocking tumor angiogenesis and metastasis. A mouse monoclonal anti-YKL-40 antibody (mAY) exhibited specific binding with recombinant YKL-40 and with YKL-40 secreted from osteoblastoma cells MG-63 and brain tumor cells U87. In the functional analysis, we found that mAY inhibited tube formation of microvascular endothelial cells in Matrigel induced by conditioned medium of MG-63 and U87 cells, as well as recombinant YKL-40. mAY also abolished YKL-40-induced activation of membrane receptor VEGF receptor 2 (Flk-1/KDR) and intracellular signaling MAP kinase Erk 1 and Erk 2. In addition, mAY enhanced cell death response of U87 line to γ-irradiation through decreased expression of pAKT and AKT, and accordingly abrogated angiogenesis induced by the conditioned medium of U87 cells in which YKL-40 levels were elevated by treatment with γ-irradiation. Furthermore, treatment of xenografted tumor mice with mAY restrained tumor growth, angiogenesis, and progression. Taken together, this study has demonstrated the therapeutic utility for the mAY in treatment of tumor angiogenesis and metastasis.
Introduction:

YKL-40 (human cartilage glycoprotein-39) is a secreted glycoprotein originally identified from the medium of a human osteosarcoma line MG-63 cells (1). Structural analyses of this 40-kDa molecule have revealed that YKL-40 is a highly phylogenetically conserved chitin-binding glycoprotein, classifying it in the family of chitinase-like proteins. However, YKL-40 lacks chitinase/hydrolase activity due to mutation of an essential glutamic acid to leucine in the chitinase-3-like catalytic domain (2, 3). Whereas the biophysiological activity of YKL-40 is poorly understood, it is believed to be associated with proliferation of connective tissue cells (4, 5) and activation of vascular endothelial cells (6). Accumulating evidence has shown that serum levels of YKL-40 were elevated in a variety of chronic inflammatory diseases (7, 8), suggestive of its pathological function being connected with the process of extracellular matrix remodeling (9, 10).

Over the last decade, particular attention has been paid to the pathologic role of YKL-40 in development of a broad type of human cancers. For instance, the database of gene microarray analyses and serial analysis of gene expression (SAGE) shows significantly higher expression levels of YKL-40 in carcinoma tissues from ovary, brain, and breast than those expressed in adjacent normal tissues (11, 12). Further, a multitude of clinical studies have found that high serum levels of YKL-40 were associated with metastasis and short survival in a number of human cancers such as breast, colorectal, ovarian, leukemia, and brain carcinoma (13-18), indicating that serum levels of YKL-40 may serve as a new cancer biomarker. While there is mounting evidence demonstrating elevated expression of YKL-40 in human cancers, little is known regarding its mechanisms underlying cancer progression.
and metastasis. Recently, we have identified YKL-40 as a tumor angiogenic factor capable of stimulating angiogenesis of microvascular endothelial cells in culture as well as in xenograft models (19). Furthermore, the expression levels of YKL-40 in human breast cancer were found to positively correlate with blood vessel formation. These findings have markedly enhanced our understanding of the molecular mechanisms of YKL-40 in the regulation of tumor angiogenesis and progression.

Tumor angiogenesis is an integral component of solid tumor growth and metastasis. Elevated levels of angiogenic factors such as VEGF and bFGF in cancer tissues directly correlate with tumor angiogenesis and tumor progression (20-22). To support these angiogenic properties, multiple complementary studies using neutralizing antibodies, including a humanized anti-VEGF antibody (bevacizumab, Avastin), showed profound impacts in restriction of tumor growth (23-25). Unfortunately, to date, functional inhibition of YKL-40 in human diseases and animal models has not been explored. Our current study sought to establish a monoclonal anti-YKL-40 antibody as a neutralizing agent for the purpose of blocking tumor angiogenesis and metastasis. Our findings highlight the potential therapeutic benefits of anti-YKL-40 treatment in patients with a wide spectrum of cancers that over-express YKL-40.
Materials and Methods:

Cell cultures: U87 cells were purchased from ATCC in October, 2008 and MG-63 cells were derived from Duke University Cell Culture Facility in September 2003. Both cell lines were kept in liquid N2 until recently grown in DMEM medium (Invitrogen) in the presence of 10% FBS. No further authentication was done during this study.

Purification of recombinant YKL-40: Full-length human YKL-40 cDNA with a 6x histidine tag was subcloned into a pFastBac1 vector (Invitrogen, CA). Following transformation and amplification in DH10Bac E. coli, bacmid DNA containing YKL-40 was transfected into Sf9 insect cells by using CellFECTIN reagent (Invitrogen) and subsequently baculoviral medium was produced. A Ni-NTA column was used to purify recombinant YKL-40 according to manufacture’s instruction (Invitrogen) and YKL-40 pure protein was finally produced through a PD-10 desalting column (Millipore, CA).

Anti-YKL-40 antibodies: rAY was generated from the immunization of rabbits with a short peptide of YKL-40 encoding C-terminus of YKL-40. Crude serum was then purified through an Econo-Pac serum IgG purification kit (BioRad, Hercules, CA). mAY was created from a hybridoma technology in which mice were initially immunized with YKL-40 recombinant protein as an antigen. Five positive hybridoma clones were screened twice with ELISA assay using recombinant YKL-40-precoated 96-well plates. Two clones with highest binding activity were further evaluated by immunoblotting and functional analysis. Finally, one clone with strong binding activity and neutralizing activity was selected and grown for the study. Its culture medium was consequently
collected and concentrated prior to purification using an Affi-gel protein A MAPS II kit (Bio-Rad).

**YKL-40 Gene knockdown:** DNA oligos (19 bp) specifically targeting YKL-40 sequences 5’ GACTCTCTTTGTCTGTCGGA 3’ (siRNA 1) or 5’ GGTGCAGTACCTGAAGGAT 3’ (siRNA 2) were selected and engineered into templates (64 oligo nucleotides) containing these oligos were subcloned into a retroviral pSUPER-puro-vector. 293T retroviral packaging cells were transfected with pSUPER siRNA constructs in the presence of pCL 10A1 vector using Fugene 6 (Roche). Forty-eight hours after transfection, the supernatant was harvested and filtered through 0.45-µm pore size filter and then the viral medium was used to infect MG63 cells. Selection with 1 µg/ml of puromycin was started 48 hr after infection and the purimycin-resistant cell populations were used for subsequent studies.

**Migration assay:** HMVECs (2 x 10^5) (26) were pre-incubated with serum-free medium overnight and then transferred onto transwells (8 µm, 24-well plates) pre-coated with collagen IV (100 µg/ml). The lower chamber of transwells included conditioned medium of MG-63 cells expressing YKL-40 siRNA or control vector. After 4 hours of incubation, HMVECs in the transwell membrane were fixed and stained. Average cell numbers were calculated from five different fields in each sample.

**Tube formation assays:** HMVEC (0.1 x 10^5 cells) were transferred onto 96-well Matrigel (BD Bioscience) in the presence of YKL-40 (100 ng/ml) or conditioned medium of tumor
cells. After 16 hours of incubation, tube-forming structures were analyzed. Averages of tubules were calculated from three fields in each sample.

**Cell viability assay:** U87 cells were treated with 10 Gy γ-irradiation in the of presence 10 μg/ml mIgG or mAY. At 48-hr culture, the cells were imaged for cell detachment. 96 hr following culture, the cells were subjected to live and dead analysis using a Live/Dead kit in which ethidium homodimer is used to detect dead cells and calcein is used to test live cells (Invitrogen Inc).

**Immunoprecipitation and Western Blot Analysis:** The samples were prepared as described before (27). The lysates were then incubated with an anti-pY20 antibody at 4°C overnight followed by incubation with protein A sepharose beads at 4°C for 4 hr. The immunocomplex was extensively washed and the samples were run on SDS-PAGE. Then proteins were transferred to a PVDF membrane (Invitrogen) and incubated with one of several antibodies: anti-YKL-40 (1:200), Flk-1/KDR (1:200), pErk1, Erk2 (1:5000, Santa Cruz), pAKT (ser473, 1:500), AKT (1:500, Cell signaling, Beverly, MA), and actin (1:1000, Sigma, MO). Specific bands were detected using an enhanced chemiluminescence kit (Pierce, VWR). Specific bands were scanned and analyzed with NIH imageJ software. Active forms of pFlk-1/KDR, pErk, and pAKT were normalized with corresponding total non-phosphorylated forms.

**Immunocytochemistry:** Cells were fixed with 4% para-formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 10 min at 4°C. Then the samples were
incubated with primary antibodies in PBS-base blocking solution containing 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20, 7.7 mM NaN₃, and 10% goat serum for one hr. Primary antibodies included purified rabbit rAY 1:100 or pre-immune serum overnight at 4°C. A secondary antibody including goat anti-rabbit Alex Fluor 488 antibody (1:1000, Invitrogen) was added for 1 hr at room temperature. Fluorescence was determined under a microscope.

**Tumor xenografts in mice:** All animal experiments were performed under the approval of Institutional Animal Care and Use Committee of the University of Massachusetts. SCID/Beige mice were subcutaneously injected with U87 cells (7.5 x 10⁶) in 0.2 ml of PBS. From week 3 when mice developed palpable tumors, mice received either a monoclonal mAY (5 mg/kg body weight) or mIgG (5 mg/kg) twice a week for 3 weeks. Tumor growth from these injected cells was monitored weekly for 5 weeks before the animals were humanely sacrificed. Tumors were measured and tumor volume was calculated as follows: volume = length x width² x 0.52.

**Immunohistochemistry:** Frozen tumor tissues from animals were cut to 6 µm thickness and processed for the staining of CD31. In brief, the samples were incubated with 3% H₂O₂ for 30 min to block endogenous peroxidase activity followed by incubation with blocking buffer containing 10% goat serum for 1 hr. Then, a rat anti-CD31 monoclonal antibody (1: 500, BD Biosciences) was incubated at room temperature for 2 hr and a goat anti-rat secondary antibody (1: 100) conjugated with HRP was added. Finally, DAB substrate (Dako Inc) was introduced for several minutes and after washing, methyl green was used for counterstaining.
Statistics: Data are expressed as mean ± SE and differences among groups were determined using one-way ANOVA analysis. The 0.05 level of probability was used as the criterion of significance.
Results

YKL-40, a secreted glycoprotein, acts as an angiogenic factor to promote tumor angiogenesis (19). To develop a monoclonal antibody as a potential neutralizing agent that can block YKL-40’s angiogenic activity, we isolated and purified 6xhistidine-tagged recombinant protein YKL-40 as an antigen for immunization of mice (Figure 1A). The purified recombinant YKL-40 retained the ability to activate endothelial cell angiogenesis \textit{in vitro} (data not shown and see below). Hybridoma cells derived from mice were grown to produce anti-YKL-40 antibody (mAY) and YKL-40-binding activity of mAY was tested using immunoblotting (Figure 1B). mAY can specifically recognize both recombinant YKL-40 and tumor-secreted YKL-40 of osteoblastoma cells MG-63 and brain tumor cells U87, both of which express YKL-40 (Figure 1B). This binding specificity was identical to a polyclonal anti-YKL-40 antibody (rAY) which was generated from rabbits immunized with a short peptide encoding c-terminus of YKL-40. As expected, mAY did not interact with samples from human microvascular endothelial cells (HMVECs) that do not express YKL-40, confirming the unique ability of mAY to react with YKL-40.

To establish an adequate \textit{in vitro} model that can recapitulate YKL-40 angiogenesis \textit{in vivo}, we employed a co-culture system including endothelial cells and tumor cells that express YKL-40. Reduced expression levels of YKL-40 from MG-63 cells were achieved through a gene knockdown approach with a stable inhibition of \textit{YKL-40}. siRNA 2, in contrast to siRNA 1, dramatically inhibited the expression of YKL-40 (Figure 2A and Supplemental Figure 1) as 30-50% of YKL-40 was reduced in siRNA2
cells compared with control or siRNA1 cells. In an attempt to characterize the angiogenic activity of YKL-40, we measured tube formation and cell migration of HMVECs, the assays that commonly recapitulate angiogenic property in vitro. Conditioned medium from MG-63 control cells and siRNA 1 cells promoted the development of tubules 4.2-fold more than control endothelial medium in the absence of the conditioned medium. Notably, YKL-40 gene knockdown of siRNA 2 reduced the tubules to 50% compared with those seen in control or siRNA 1 cells (Figure 2B). Consistent with this result, these conditioned media gave rise to the same effect on cell migration as the tube formation (Figure 2C), revealing a paracrine effect of YKL-40 in the induction of angiogenesis in vivo. To determine the ability of mAY to suppress the angiogenic activity of YKL-40, we utilized the tube formation assay and found that mAY fully inhibited tubules induced by the conditioned medium of MG-63 cells (Figure 3A). Likewise, mAY abolished the tubules developed by conditioned medium of U87 cells in a dose-dependent manner (Figure 3B). In addition, mAY also blocked angiogenesis induced by recombinant protein YKL-40 (Figure 3C and Supplemental Figure 2), validating the neutralization of mAY on the angiogenic property of YKL-40 present in tumor cell-conditioned medium. All of this data demonstrates that mAY acts as a neutralizing antibody to effectively inhibit YKL-40-induced angiogenesis in vitro.

In order to explore mechanisms of mAY in the inhibition of YKL-40-induced angiogenesis, we examined expression of VEGF receptor 2 (Flk-1/KDR) in endothelial cells, one of the most important membrane-associated tyrosine kinase receptors which mediate endothelial cell angiogenesis (27). Treatment of HMVECs with recombinant
YKL-40 resulted in elevated expression of Flk-1/KDR in a dose- and time-dependent manner (Figure 4A). Likewise, this induction was also observed in the cells treated with U87 conditioned medium, but it was diminished in the presence of mAY (Figure 4A). Interestingly, YKL-40 also notably induced tyrosine phosphorylation of Flk-1/KDR and downstream effector MAP kinase pErk1 and pErk2 (Figure 4B and 4C). However, treatment with mAY significantly reduced pFlk-1/KDR, pErk1, and pErk2 (Figure 4B and 4C), as pFlk-1 reduced to basal levels and pErk 1 and pErk 2 were reduced by 40-45% compared to control or mIgG treatment, suggesting an interruption of the angiogenic signaling cascades through Flk-1/KDR to MAPK Erk 1 and Erk 2. To test whether or not Erk is the only one downstream effector of Flk-1/KDR, we treated the HMVECs with Flk-1/KDR kinase inhibitor SU1498 (12.5 μM) and found that the blockade of Flk-1/KDR activity failed to suppress Erk phosphorylation induced by recombinant protein YKL-40 (100 ng/ml) (data not shown). The data suggest that other upstream mediators of Erk apart from Flk-1/KDR also regulate its activation.

It was established that serum levels of YKL-40 were elevated in cancer patients treated with radiation therapy. Furthermore, these elevated concentrations positively correlated with cancer recurrence and poor survival, suggesting that serum levels of YKL-40 serve as a prognostic biomarker (13-18). To test the hypothesis that γ-irradiation-induced YKL-40 protects tumor cell death, we monitored the levels of YKL-40 in U87 cells exposed to γ-irradiation and measured cell survival in the presence of mAY. As anticipated, exposure of the cells to γ-irradiation resulted in increased concentrations of YKL-40 (Figure 5A). Once these cells were simultaneously treated
with mAY for 48 hr, they displayed a phenotype indicative of failure to survive, as more than 80% of these cells were detached from culture plates compared with the counterparts that showed 30% of detached cells in the absence of mAY (Figure 5B). To assess whether these cells undergo apoptosis, culture was extended to 96 hr and mAY treatment led to cell death around 1.5-fold greater than control or mIgG treated cells (Figure 5B). To further determine whether the PI3K-AKT signaling cascade mediates the YKL-40-induced cell survival activity, we collected cell lysates and measured the expression of these proteins using immunoblotting. Intriguingly, mAY treatment led to decreases in activated AKT and non-activated AKT by 65-82%, but did not affect PI3K expression (Figure 5C) or kinase activity (data not shown), suggesting that YKL-40-mediated cell survival is AKT-dependent, but PI3K-independent. We next questioned whether the up-regulation of YKL-40 by γ-irradiation is capable of prompting endothelial cell angiogenesis through a paracrine fashion, which may support tumor cell survival in vivo. To address this, we collected the conditioned medium of U87 cells treated with γ-irradiation and transferred to HMVECs for the tube formation assay. The medium from γ-irradiation-treated cells increased tubules two-fold more than did the control medium (Figure 5D). However, mAY completely abrogated the angiogenic effects of YKL-40 in HMVECs, strongly suggesting that mAY be used as an efficacious agent for the treatment of radioresistant cancers.

Finally, we sought to explore the inhibitory effects of mAY on tumor growth and angiogenesis induced by YKL-40 in vivo. SCID/Beige mice were subcutaneously injected with U87 cells and once these mice developed palpable tumors by week 3, they
were treated with either mAY or mIgG as a control (5 mg/kg body weight, subcutaneously) twice a week for two weeks. As demonstrated in Figure 6A, treatment of mice with mAY significantly inhibited tumor growth by approximately 40% relative to mIgG treatment. To determine whether anti-angiogenic activity of mAY contributes to the tumor suppression, we examined expression of CD31, a vascular endothelial cell marker in tumor samples (Figure 6B). Histological analysis exhibited a remarkable reduction in blood vessel formation in tumors from mice treated with mAY, as vessel density of mAY-treated tumors was only 28% that of the control group. In parallel with this inhibited tumor angiogenesis, decreased incidence of ectopic tumors was also observed (Figure 6C). Two of five (40%) mice treated with mAY displayed the normal liver free from tumor cells, one mouse (20%) developed sparse liver metastases, and the remainder (40%) exhibited extensive liver metastasis. In contrast, all mIgG-treated control mice (100%) developed massive liver metastasis (Figure 6C). No lung metastasis was identified in either group (data not shown). Taken together, these in vivo results support the notion that mAY may serve as a powerful agent for the suppression of tumor angiogenesis and metastasis in a variety of advanced cancers that over-express YKL-40.
Discussion:

Our current study has established a monoclonal antibody specific for YKL-40 and demonstrated that it can neutralize YKL-40 function both in vitro and in vivo. This finding offers immense value for the future development of an anti-YKL-40 humanized antibody not only in the treatment of cancer patients but also in the therapy of other diseases. Serum levels of YKL-40 have been long viewed as a biomarker in various types of human diseases including cancers (15, 18, 28), inflammatory diseases (9), hepatic fibrosis (8), and atherosclerosis (29). Furthermore, these increased concentrations of YKL-40 positively correlate with the progression of these diseases, indicative of a pathologic role played by YKL-40. We have recently identified that YKL-40 acts as a novel angiogenic factor to stimulate vascular endothelial cell migration and vasculature generation in cancer, independent of VEGF activity (19). In concert with these published data, here we found that YKL-40 secreted from U87 and MG-63 cells and recombinant YKL-40 possess the same ability to promote endothelial cell angiogenesis in vitro. However, mAY fully blocked this angiogenic activity, strongly suggesting that the YKL-40 neutralizing antibody serves as a therapeutic agent for the treatment of cancers and other diseases that over-produce YKL-40.

While mAY showed a neutralizing activity on angiogenesis in vitro and in vivo, it failed to completely abolish tumor growth in our xenografted model. This inconsistent result may be attributed to a number of possibilities that occur in animal models. First, we started the treatment of animals with mAY from week 3 when tumors had established
significant tumor mass that poorly responded to mAY. Second, the dose of mAY we used (5 mg/kg body weight) in this study may be insufficient to fully eliminate YKL-40 activity, as ten times of anti-VEGF receptor antibody (DC101) or double amount of anti-VEGF neutralizing antibody (bevacizumab) were tested by other groups in xenograft tumor models previously (30, 31). In addition, it is likely that a variety of angiogenic factors other than YKL-40 such as bFGF which are produced by tumor cells, also mediate vasculature formation in vivo (in our unpublished data). Finally, host tissue may play a role in facilitating angiogenesis as well because YKL-40 and other growth factors could be derived from a number of infiltrating cells including macrophages and neutrophils when tumors develop and invade adjacent normal tissue (32, 33). Thus, mAY is incapable of blocking other pro-angiogenic and/or tumor-promoting factors that contribute to tumor development. Nonetheless, our study has demonstrated the effectiveness of mAY in inhibition of tumor angiogenesis and growth.

The evidence that tumor cell-conditioned medium exhibited more pronounced impacts on tube formation than did recombinant YKL-40 (Figure 3) strongly suggests that other potential angiogenic factors derived from tumor cells may also participate in tumor angiogenesis. However, this angiogenesis induced by the conditioned medium can be sufficiently blocked by mAY, implicating a central role that YKL-40 plays in regulating other angiogenic factors. Indeed, we found in a separate study that YKL-40 up-regulates VEGF expression in U87 cells (Francescone et al., manuscript submitted). Interestingly, mAY is able to fully eliminate tube formation in the presence of tumor cell-conditioned medium, in contrast to its inhibition in the presence of recombinant YKL-40,
suggesting that blockade of YKL-40 in the conditioned medium also induces the release of other factors from tumor cells which may profoundly impair tube development.

Our previous study has found that YKL-40 induced coordination of membrane-bound receptor syndcan-1 and integrin αvβ3 and activated intracellular signaling cascade, including FAK, Erk 1 and Erk 2 (19). Here, we identified that YKL-40 not only increased expression of Flk-1/KDR, a VEGF receptor 2 that mediates VEGF angiogenesis (27) but also activated the tyrosine phosphorylated form of Flk-1/KDR, possibly leading to a synergistic effect on the angiogenic signaling activation. Erk 1 and Erk 2 were found to be the downstream intracellular effectors. Notably, mAY abrogated all these signaling cascades induced by YKL-40. These data demonstrate the molecular mechanisms underlying YKL-40-induced angiogenic responses in endothelial cells and underscore the neutralizing activity of mAY in the inhibition of angiogenesis. It remains to be determined whether YKL-40-induced tyrosine phosphorylation of Flk-1/KDR is dependent on or independent of activation of other adjacent membrane receptor S1 and integrin αvβ3.

We found that γ-irradiation induced YKL-40 expression which not only protected cell death, but also elicited endothelial cell angiogenesis through a paracrine loop. We found that mAY sensitized the death responses of tumor cells to γ-irradiation through a decrease of PI3K-independent AKT phosphorylation, a common survival pathway which
has been established previously (34-36). Consistent with our results, MAPK and AKT were reported to mediate YKL-40-induced mitogenic signaling in connective tissue cells (4). In addition, we interestingly found that mAY nullified endothelial cell angiogenesis induced by U87 conditioned medium treated with γ-irradiation. Therefore, our studies offered mechanistic insight into radioresistance of cancers that express increased levels of YKL-40 and demonstrate poor prognosis (18, 37). Furthermore, our findings shed light on the pronounced impacts of YKL-40 neutralizing antibody on cancer progression in conjunction with radiation therapy.

In current cancer therapy, particular attention has been focused on a number of anti-angiogenic drugs approved by FDA such as anti-VEGF antibody bevacizumab and VEGF receptor tyrosine kinase inhibitors (sorafenib and sunitinib). However, the benefits of these anti-angiogenic agents appear to be transitory in the treatment of several types of advanced cancers, as drug resistance, tumor regrowth, and extensive vascular recovery rapidly develop once the therapy is terminated (38-40). Furthermore, there are several recent studies showing the opposite effects of these anti-angiogenic drugs on tumor growth, angiogenesis, and metastasis in xenografted tumor models. For example, treatment of xenotransplant models with an anti-VEGF receptor 2 antibody unexpectedly resulted in extensive tumor revascularization, increased invasiveness, and rapidly ectopic dissemination (30, 41). In line with this evidence, a short-term therapy with sunitinib and SU11248 (VEGF and PDGF receptor kinase inhibitor) accelerated local tumor invasion and multiple distant metastases after intravenous injection of tumor cells or removal of primary tumors (42). This immediately acquired adaptation to the anti-angiogenic
therapies is believed to be associated with the angiogenic switch by which tumors undergo robust revascularization and malignant transformation (41). It would be quite interesting to know whether YKL-40 plays an active role in these evasion responses. Nevertheless, the combined anti-angiogenic approaches that include anti-angiogenic monoclonal antibodies and membrane receptor kinase inhibitors together with other chemotherapies pave the way towards a more efficacious therapy for cancer patients.
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Antiangiogenic therapy elicits malignant progression of tumors to increased local


Figure legends:

**Figure 1.** Anti-YKL-40 antibodies recognize both YKL-40 protein secreted from tumor cells and purified recombinant YKL-40.  
**A.** Recombinant YKL-40 was generated by a baculoviral system. His-tagged YKL-40 was purified through a Ni-NTA affinity-binding column followed by a desalting PD-10 column. Collected samples were analyzed by immunoblotting with an anti-6xhistidine antibody.  
**B.** YKL-40 levels in cell conditioned medium collected from MG-63 cells, U87 cells, and HMVECs as well as recombinant YKL-40 were detected by immunoblotting using rAY and mAY.

**Figure 2.** YKL-40 siRNA inhibits endothelial cell angiogenesis induced by YKL-40.  
**A. Western blot.** Conditioned media from MG-63 expressing siRNA1, siRNA2 and vector control were measured for YKL-40, and cell lysates were used for actin using immunoblotting followed by quantitative analysis. Intensity of YKL-40 and actin was analyzed with NIH ImageJ software and YKL-40 levels were normalized with actin expression. Subsequently, these levels were compared with the basal control arranged as one unit.  
**B. Tube formation.** DMEM serum-free medium of MG-63 cells expressing YKL-40 siRNA were collected after 24 hr and transferred to Matrigel. After 16-hr incubation, tubules were imaged and tube density was analyzed quantitatively. n=5. Control represents DMEM serum-free medium and MG-63 control indicates DMEM serum-free conditioned medium of MG-63 control cells.  
**C. Cell migration.** Same conditioned medium of MG-63 cells as described above was transferred to the bottom chamber of a transwell in order to test its effects on migration of HMVEC that were loaded onto the top chamber of the well. After 4-hr incubation, migrated cells were fixed.
and stained. The quantification was shown in the bottom panel. n=4. *P<0.05 compared with DMEM serum-free medium as controls, and †P<0.05 compared with MG-63 control or siRNA1. Bars: 100 μm.

Figure 3. **mAY blocks tube formation induced by cell conditioned medium containing YKL-40.** A. **mAY inhibits tube formation induced by both conditioned medium of MG-63 and U87 cells.** MG-63 and U87 cells were pretreated with mAY or mIgG (10 μg/ml) for 24 hr and the conditioned medium was transferred to HMVECs for the tube formation. The data were quantified. n=3. B. **mAY-inhibited tube formation is dose-dependent.** Tube formation of HMVEC was determined in the presence of conditioned medium of U87 cells pretreated with mAY at 5, 10, 20 μg/ml or mIgG at 20 μg/ml for 24 hr. The data were quantified. *P<0.05 compared mIgG. n=4. C. **mAY blocks tube formation induced by recombinant YKL-40.** mAY or mIgG (10 μg/ml) was introduced to serum-free medium of HMVECs in the presence of recombinant YKL-40 from 50-250 ng/ml in Matrigel and quantification of tube formation was displayed. *P and †P<0.05 compared with non-YKL-40-treated controls and corresponding mIgG treatments, respectively. n=4. Bars: 100 μm.

Figure 4. **mAY interrupts Flk-1/KDR and MAPK Erk signaling pathways induced by YKL-40.** A. **mAY blocks YKL-40-induced Flk-1/KDR expression.** HMVECs pretreated with serum-free medium overnight were stimulated with 100 ng/ml recombinant protein YKL-40 from 0 to 40 hr or with 0-200 ng/ml YKL-40 for 24 hr. Conditioned medium
from U87 cells in the presence or absence of 10 μg/ml mIgG or mAY was transferred to HMVECs for 24 hr as described in Figure 3A. Subsequently, all of the cell lysates were tested for Flk-1/KDR expression by immunoblotting. Flk-1/KDR data were normalized with actin levels and then compared with the basal serum-free level arranged as one unit. n=4, mean ± SE.  

**B. mAY blocks YKL-40-induced tyrosine phosphorylation of Flk-1/KDR.** HMVECs were pretreated with serum-free medium overnight in the presence of mAY or mIgG (10 μg/ml) and then directly stimulated with recombinant YKL-40 (100 ng/ml) for 10 min. n=2, mean ± SE. For treatment with cell conditioned medium, HMVECs were pretreated with serum-free medium overnight and then treated for 10 min with U87 cell conditioned medium that was exposed to mAY or mIgG (10 μg/ml) for 24 hr. Half of the cell lysates were immunoprecipitated with an antibody against phosphorylated tyrosine protein (pY20) followed by immunoblotting using an antibody against Flk-1/KDR. The remaining lysates were used directly for immunoblotting against total Flk-1/KDR. IgG of the anti-pY20 antibody was also tested as loading controls. pFlk-1/KDR was normalized with total Flk-1/KDR. n=3, mean ± SE.  

**C. mAY blocks YKL-40-induced MAPK Erk 1 and Erk 2 activation.** The same conditions of HMVECs as described in B were collected and immunoblotted with antibodies against pErk 1, Erk 2, and total Erk followed by quantification. pErk 1 and pErk 2 levels were normalized with total Erk 1 and Erk 2. SF indicates serum-free basal DMEM and Con represents DMEM serum-free conditioned medium alone. n=2, mean ± SE.  

**Figure 5.** mAY enhances tumor cell death responses to γ-irradiation and decreases endothelial cell angiogenesis.  

**A. γ-irradiation induced YKL-40 expression.** U87 cells
were treated with 0, 3, 5, or 10 Gy of \(\gamma\)-irradiation and 48 hr later, cell conditioned medium was subjected to testing YKL-40 levels and cell lysates were examined for actin levels. YKL-40 levels were quantified by normalization with actin. The basal level of YKL-40 without \(\gamma\)-irradiation was set as one unit. \(n=2\), mean ± SE. **B. mAY decreases cell viability in response to \(\gamma\)-irradiation.** In the upper panel, U87 cells treated with 10 Gy \(\gamma\)-irradiation as described above in the presence of mAY or mIgG (10 \(\mu\)g/ml) were determined for cell viability in cultured condition. Attached and detached cells were quantified under a microscope. In the bottom panel, 96 hr following treatment with 10 Gy \(\gamma\)-irradiation above, U87 cells were assayed for cell death as described in the Method. Red fluorescence indicates dead cells and green fluorescence represents live cells. *\(P<0.05\) compared with the control or mIgG-treated group. \(n=5\). Bars: 50 \(\mu\)m. **C. mAY decreases pAKT and AKT levels.** U87 cells treated with \(\gamma\)-irradiation for 48 hr as described in B were tested for pAKT (ser473), AKT, PI3K, and actin. pAKT and AKT levels were normalized with actin and subsequently, these levels were compared with their basal expressions without \(\gamma\)-irradiation arranged as one unit. \(n=2\), mean ± SE. **D. mAY blocks \(\gamma\)-irradiation-induced angiogenesis.** Conditioned medium of U87 cells as described in C was transferred to HMVECs for testing tubules in Matrigel. \(n=4\). *\(P<0.05\) compared with cells without \(\gamma\)-irradiation and +\(P<0.05\) compared with control or mIgG.

**Figure 6. mAY inhibits tumor growth, angiogenesis and progression.** **A.** U87 cells were injected subcutaneously into SCID/Beige mice and mAY or mIgG (5 mg/kg body weight) was injected subcutaneously twice a week from week 3. Tumor volume was evaluated,
n=5. *P<0.05 compared with corresponding mIgG. **B.** Tumor samples were collected and subjected to IHC of CD31 staining. Brown color indicates positive staining for blood vessels. Bar: 100 μm. Vessel density was quantified using ImageJ software. *P<0.05 compared with corresponding mIgG. **C.** Liver was removed from mice and processed for H & E staining. Dark blue cells are tumor cells. Bar: 100 μm.
Fig. 1
**Fig. 3**

(A) Tube formation assay for MG-63 and U87 cells treated with mlgG and mAY.

(B) Tube formation assay for MG-63 and U87 cells treated with different concentrations of mlgG and mAY.

(C) Graph showing the effect of recombinant MKL-4 on tube formation.
**Figure 6**

(A) Graph showing tumor volume over time with two lines representing different treatments: IgG and mAY.

(B) Micrographs showing vessel density with mlG and mAY treatments, and a bar graph depicting vessel density quantification.

(C) Micrographs showing liver metastasis with mlG and mAY treatments, and a bar graph depicting liver metastasis percentage.
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