A YKL-40–Neutralizing Antibody Blocks Tumor Angiogenesis and Progression: A Potential Therapeutic Agent in Cancers

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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 the membrane receptor VEGF receptor 2 (Flk-1/KDR) and intracellular signaling mitogen-activated protein (MAP) kinase extracellular signal–regulated 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 shown the therapeutic use for the mAY in treatment of tumor angiogenesis and metastasis. Mol Cancer Ther; 10(5); 742–51. ©2011 AACR.

Introduction

The human cartilage glycoprotein-39 (YKL-40) is a secreted glycoprotein originally identified from the medium of a human osteosarcoma cell line, MG-63 (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 biophysicologic 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 pathologic 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). Furthermore, 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. Although there is mounting evidence showing 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 basic fibroblast growth factor (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 American Type Culture Collection in October 2008, and MG-63 cells were derived from Duke University Cell Culture Facility, Durham, NC, in September 2003. Both cell lines were kept in liquid N2 until recently grown in Dulbecco’s Modified Eagle’s 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 6xhistidine tag was subcloned into a pFastBac1 vector (Invitrogen). Following transformation and amplification in DH10Bac E. coli, bacmid DNA containing YKL-40 was transfected into Sf9 insect cells by using Cellfectin reagent (Invitrogen). 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. An Ni-NTA column was used to purify recombinant YKL-40 according to manufacturer’s instruction (Invitrogen) and YKL-40 pure protein was finally produced through a PD-10 desalting column (Millipore).

Anti–YKL-40 antibodies

Polyclonal anti–YKL-40 (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). 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, 1 clone with strong binding activity and neutralizing activity was selected and grown for the study. Its culture medium was consequently collected and concentrated before 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’ GACTCTCTTTGTCTGCGGA 3’ [short interfering RNA (siRNA) 1] or 5’ GGTCCAGTACCTGAGATT 3’ (siRNA 2) were selected and engineered into templates (64 oligonucleotides) 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 hours after infection and the puromycin-resistant cell populations were used for subsequent studies.

Migration assay

Human microvascular endothelial cells (HMVEC; 2 × 10^5; ref. 26) were preincubated with serum-free medium overnight and then transferred onto Transwell (8-μm, 24-well plates) precoated with collagen IV (100 μg/mL). The lower chamber of Transwell included conditioned medium of MG-63 cells expressing YKL-40 mRNA or control vector. After 4 hours of incubation, HMVECs in the Transwell membrane were fixed and stained. Average tube numbers were calculated from 5 different fields in each sample.

Tube formation assays

HMVECs (0.1 × 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 tubes were calculated from 3 fields in each sample.

Cell viability assay

U87 cells were treated with 10 Gy γ-irradiation in the presence of 10 μg/mL mlgG or mAY. At 48-hour culture, the cells were imaged for cell detachment. Ninety-six hours 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).

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 hours. The immunocomplex was extensively washed and the samples were run on SDS-PAGE. Then proteins were transferred
to a PVDF (polyvinylidene difluoride) membrane (Invitrogen) and incubated with one of several antibodies: anti-YKL-40 (1:200), Flk-1/KDR (1:200), phosphorylated form of extracellular signal–regulated kinase (pErk) 1, Erk 2 (1:5,000; Santa Cruz), pAKT (ser473, 1:500), AKT (1:500, Cell Signaling Technology), and actin (1:1,000; Sigma). 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 nonphosphorylated forms.

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.5% Triton X-100 for 10 minutes at 4°C. Then, the samples were incubated with primary antibodies in PBS-base blocking solution containing 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween-20, 7.7 mmol/L NaNO3, and 10% goat serum for one hour. Primary antibodies included purified rabbit rAY 1:100 or preimmune serum overnight at 4°C. A secondary antibody including goat anti-rabbit Alexa Fluor 488 antibody (1:1,000; Invitrogen) was added for 1 hour at room temperature. Fluorescence was determined under a microscope.

**Tumor xenografts in mice**

All animal experiments were carried out under the approval of Institutional Animal Care and Use Committee of the University of Massachusetts. Severe combined immunodeficient mice (SCID)/Beige mice were subcutaneously injected with U87 cells (7.5 × 106) in 0.2 mL of PBS. From week 3, when mice developed palpable tumors, mice received either a mAY (5 mg/kg body weight) or mlgG (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 × width² × 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 minutes to block endogenous peroxidase activity followed by incubation with blocking buffer containing 10% goat serum for 1 hour. Then, a rat anti-CD31 monoclonal antibody (1:500, BD Biosciences) was incubated at room temperature for 2 hours and a goat anti-rat secondary antibody (1:1,000) conjugated with horseradish peroxidase was added. Finally, DAB (3,3′-diaminobenzidine) substrate (Dako) was introduced for several minutes and after washing, methyl green was used for counterstaining.

**Statistics**

Data are expressed as mean ± standard error (SE) and differences among groups were determined using 1-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 angiogenic activity of YKL-40, we isolated and purified 6x histidine-tagged recombinant protein YKL-40 as an antigen for immunization of mice (Fig. 1A). The purified recombinant YKL-40 retained the ability to activate endothelial cell angiogenesis in vitro (data not shown and see later). 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 (Fig. 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 (Fig. 1B). This binding specificity was identical to a 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 HMVECs that do not express YKL-40, confirming the unique ability of mAY to react with YKL-40.

To establish an adequate in vitro model that can recapitulate YKL-40 angiogenesis in vivo, we used a coculture system consisting of 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 YKL-40. siRNA 2, in contrast to siRNA 1, dramatically inhibited the expression of YKL-40 (Fig. 2A and Supplementary Fig. S1) as 30% to 50% of YKL-40 was reduced in siRNA 2 cells compared with control or siRNA 1 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 (Fig. 2B). Consistent with this result, these conditioned media gave rise to the same effect on cell migration as the tube formation (Fig. 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 used the tube formation assay and found that mAY fully inhibited tubules induced by the conditioned medium of MG-63 cells (Fig. 3A). Likewise, mAY abolished the tubules developed by conditioned medium of U87 cells in a dose-dependent manner (Fig. 3B). In addition, mAY also blocked angiogenesis induced by recombinant protein YKL-40 (Fig. 3C and Supplementary Fig. S2), validating the neutralization of mAY on the angiogenic property of YKL-40 present in tumor cell conditioned medium. All of these data show that mAY acts as a neutralizing antibody to effectively inhibit YKL-40–induced angiogenesis in vitro.

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 (Fig. 4A). Likewise, this induction was also observed in the cells treated with U87 conditioned medium, but it was diminished in the presence of mAY (Fig. 4A). Interestingly, YKL-40 also notably induced tyrosine phosphorylation of Flk-1/KDR and downstream effector mitogen-activated protein (MAP) kinase pErk 1 and pErk 2 (Fig. 4B and C). However, treatment with mAY significantly reduced pFlk-1/KDR, pErk 1, and pErk 2 (Fig. 4B and C), as pFlk-1 reduced to basal levels and pErk 1 and pErk 2 were reduced by 40% to 45% compared with 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 μmol/L) 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
Corresponding mIgG treatments, respectively. YKL-40 may or mIgG (10 n quantified. The data were determined in the presence of conditioned medium of U87 cells pretreated with mAY or mIgG (10 µg/mL) for 24 hours and the conditioned medium was transferred to HMVECs for the tube formation. The data were quantified. *, *P < 0.05 compared mIgG. n = 4. 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 hours 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, and 20 µg/mL for 24 hours. The data were quantified. n = 4. C, mAY blocks tube formation induced by recombinant YKL-40 (250 ng/mL) for 24 hours and the 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 hours 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, and 20 µg/mL or mIgG at 20 µg/mL for 24 hours. 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 containing YKL-40 from 50 to 250 ng/mL in Matrigel and quantification of tube formation was displayed. *, *P < 0.05 compared with non-YKL-40-treated controls and corresponding mIgG treatments, respectively, n = 4. Bars, 100 µm.

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 (Fig. 5A). Once these cells were simultaneously treated with mAY for 48 hours, 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 (Fig. 5B). To assess whether these cells undergo apoptosis, culture was extended to 96 hours and mAY treatment led to cell death around 1.5-fold greater than control or mIgG-treated cells (Fig. 5B). To further determine whether the phosphoinositide 3-kinase (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 nonactivated AKT by 65% to 82%, but did not affect PI3K expression (Fig. 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 upregulation 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 2-fold more than did the control medium (Fig. 5D). However, mAY completely abrogated the angiogenic effects of YKL-40 in HMVECs, strongly suggesting that mAY may be used as an efficacious agent for the treatment of radiotherapy-resistant 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 2 weeks. As shown in Fig. 6A, treatment of mice with mAY significantly inhibited tumor growth by approximately 40% relative to mIgG treatment. To determine whether antiangiogenic activity of mAY contributes to the tumor suppression, we examined expression of CD31, a vascular endothelial cell marker in tumor samples (Fig. 6B). Histologic 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 (Fig. 6C). Two of 5 (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 metastases. In contrast, all mIgG-treated control mice (100%) developed massive liver metastases (Fig. 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 overexpress YKL-40.
Discussion

Our current study has established a monoclonal antibody specific for YKL-40 and showed 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 overproduce YKL-40. Although 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 10 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 proangiogenic and/or tumor-promoting factors that contribute to tumor development. Nonetheless, our study has shown 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 (Fig. 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 mAY nullified endothelial cell angiogenesis induced by U87 conditioned medium treated with γ-ray irradiation. Therefore, our studies offer mechanistic insight into radiotherapy resistance of cancers that express increased levels of YKL-40 and show poor prognosis (18, 38). 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 antiangiogenic drugs approved by FDA (Food and Drug Administration) such as anti-VEGF antibody bevacizumab and VEGF receptor tyrosine kinase inhibitors (sorafenib and sunitinib). However, the benefits of these antiangiogenic 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 (39–41). Furthermore, there are several recent studies showing the opposite effects of these antiangiogenic 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, 42). In line with this evidence, a short-term therapy with sunitinib and SU11248 (VEGF and platelet-derived growth factor receptor kinase inhibitor) accelerated local tumor growth.

Our previous study has found that YKL-40 induced coordination of membrane-bound receptor syndecan 1 and integrin αvβ3 and activated intracellular signaling cascade including FAK (focal adhesion kinase), 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 show 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 or independent of activation of other adjacent membrane receptor syndecan1 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 (35–37). 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 radiotherapy resistance of cancers that express increased levels of YKL-40 and show poor prognosis (18, 38). Furthermore, our findings shed light on the pronounced impacts of YKL-40-neutralizing antibody on cancer progression in conjunction with radiation therapy.
invasion and multiple distant metastases after intravenous injection of tumor cells or removal of primary tumors (43). This immediately acquired adaptation to the antiangiogenic therapies is believed to be associated with the angiogenic switch by which tumors undergo robust revascularization and malignant transformation (42). It would be quite interesting to know whether YKL-40 plays an active role in these evasion responses. Nevertheless, the combined antiangiogenic approaches that include antiangiogenic monoclonal antibodies and membrane receptor kinase inhibitors together with other chemotherapies pave the way toward a more efficacious therapy for cancer patients.

## References


Molecular Cancer Therapeutics

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