YKL-40 secreted from adipose tissue inhibits degradation of type I collagen

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ABSTRACT

Obesity is considered a chronic low-grade inflammatory status and the stromal vascular fraction (SVF) cells of adipose tissue (AT) are considered a source of inflammation-related molecules. We identified YKL-40 as a major protein secreted from SVF cells in human visceral AT. YKL-40 expression levels in SVF cells from visceral AT were higher than in those from subcutaneous AT. Immunofluorescence staining revealed that YKL-40 was exclusively expressed in macrophages among SVF cells. YKL-40 purified from SVF cells inhibited the degradation of type I collagen, a major extracellular matrix of AT, by matrix metalloproteinase (MMP)-1 and increased rate of fibril formation of type I collagen. The expression of MMP-1 in preadipocytes and macrophages was enhanced by interaction between these cells. These results suggest that macrophage/preadipocyte interaction enhances degradation of type I collagen in AT, meanwhile, YKL-40 secreted from macrophages infiltrating into AT inhibits the type I collagen degradation.

Key words: YKL-40, MMP-1, type I collagen, adipose tissue, macrophage, preadipocyte

Introduction

Adipose tissue (AT) is a critical exchange center for complex energy transactions involving triacylglycerol storage and release. It also has an active endocrine role, releasing various adipokines that participate in complex pathways to maintain metabolic and vascular health [1]. At present, obesity is considered a low-grade inflammatory status, a feature shared with associated pathologies like type 2 diabetes and atherosclerosis [2,3]. AT is composed of mature adipocytes and stromal vascular fraction (SVF) cells which include preadipocytes, fibroblasts, endothelial cells, infiltrating macrophages, *etc*. Several experiments suggest that some inflammatory factors mostly originate from the non-adipocyte fraction, including infiltrating macrophages [4,5]. The amount of infiltrating macrophages that secrete inflammatory cytokines such as TNF-α, IL-1α, β, and IL-6 is closely related with AT mass [6,7]. Preadipocytes in SVF cells have been reported to act as macrophage-like cells and secrete inflammatory cytokines [8] and mediate lipopolysaccharide-induced inflammation and insulin resistance [9]. Thus, SVF cells including macrophages and preadipocytes are thought to be causes of inflammatory status in AT.

To identify proteins secreted from human SVF cells, we attempted a proteomic analysis of the conditioned medium (CM) of SVF cells fractionated from human AT. We found that YKL-40 (also known as HC gp-39 and chitinase 3-like protein 1) is a major protein in CM of SVF cells from visceral AT. YKL-40, a member of the mammalian chitinase-like proteins, is expressed and secreted by human synovial cells [10,11], chondrocytes [11], smooth muscle cells [12], neutrophils [13], and macrophages [14]. Although YKL-40 is classified as a member of glycoside hydrolase family 18, which includes bacterial and plant chitinases and retains the capacity to bind chitin, it has no chitinase activity [11]. Increased plasma levels of YKL-40 have been

found in patients with various pathologies, including arthritis [15], liver fibrosis [15], various tumors [15,16], type 2 diabetes [17], and asthma [18]. Recently, YKL-40 derived from bovine nasal cartilage and chondrocytes was reported to bind type I collagen and modulate the rate of type I collagen fibril formation [19]. These reports indicate roles for YKL-40 in inflammation and connective tissue remodeling. However, the exact function of YKL-40 is yet to be uncovered. In this study, we examined the functions of human YKL-40 secreted from macrophages in AT.

Materials and methods

Subjects. Fully informed consent was obtained from each patient in accordance with institutional guidelines. The study was approved by our internal review board. Seventeen subjects with various tumors participated. Paired visceral (the omentum) and subcutaneous (the abdominal wall) ATs were biopsied from these subjects during resection of tumors. Clinical data are presented in Supplementary Table 1.

Fractionation of AT. Visceral or subcutaneous AT was minced and washed with PBS containing 2% BSA. The tissues were digested in minimum essential medium (WAKO, Tokyo, Japan) containing 1 mg/ml collagenase S1 (Nitta-Gelatin, Osaka, Japan) with shaking at 37˚C for 30 min. The samples were filtered through a nylon mesh and centrifuged at 700 x *g* for 2 min. The precipitated SVF cells were washed with Ham's F12 medium (WAKO) containing 10% newborn calf serum (NCS; Invitrogen, Carlsbad, CA) three times. SVF cells were cultured in collagen gels (Cellmatrix Collagen Gel Culturing Kit; Nitta-Gelatin) with Ham's F12 medium containing 10% NCS on 6-well plates.

Mass spectrometry. SVF cells in collagen gels were incubated with serum–free DMEM (WAKO) for 24 h. The CM was precipitated with cold acetone and the pellet after centrifugation was dissolved in PBS containing 0.1% Triton X-100. The samples were subjected to SDS-PAGE, followed by CBB-staining. Visible bands were excised and digested in-gel with Trypsin Gold Mass Spectrometry Grade (Promega, Madison, WI). These samples were desalted using ZipTipC18 pipette tips (Millipore, Bedford, MA). Mass spectrometry of the samples was performed by MALDI-TOF-MS (MS/MS 4700 proteomics discovery system; Applied Biosystems, Foster City, CA).

The MASCOT peptide mass fingerprint online search engine (http://www.matrixscience.com) was used to identify the samples from the NCBI non-redundant data-base.

Cell culture. SVF cells isolated from human visceral or subcutaneous AT were cultured with Ham's F12 medium supplemented with 10% NCS. The cells were used for experiments after at least three passages in serum-containing medium and were defined as passaged preadipocytes, because preadipocytes tended to grow more preferentially than other cell types during passage culture of SVF cells. Human monocytic cell lines, THP-1 and U937, were cultured in RPMI 1640 medium (WAKO) supplemented with 10% FBS and differentiated into macrophages by 24 h of incubation in 100 ng/ml phorbol 12-myristate 13-acetate (Sigma, St Louis, MO). The macrophage-CM was collected after 24 h of incubation with serum-free DMEM.

RNA extraction and real-time RT-PCR. Total RNA was isolated from adipocytes, preadipocytes, and macrophages differentiated from THP-1 or U937 cells, using ISOGEN (Nippongene, Tokyo, Japan). The total RNA of passaged preadipocytes incubated for 24 h with serum-free DMEM with or without 50% macrophage-CM was extracted in the same manner. Each cDNA was prepared by reverse transcription using a PrimeScript™ RT Reagent Kit (TaKaRa, Shiga, Japan). The mRNA levels of various proteins were analyzed by SYBR green quantitative real-time RT-PCR with a specific primer set (see Supplementary Table 2) using a 7300 real-time PCR system (Applied Biosystems). The mRNA level of each target gene was normalized using GAPDH as an internal control.

Immunofluorescence staining. The cells were cultured on 6-well plates and rinsed

in PBS and then fixed in 10% formaldehyde. Fixed cells were incubated first with 0.1% Triton X-100 for 10 min followed by blocking solution (Blocking One: Nacalai Tesque, Kyoto, Japan) for 1 h. The cells were incubated with a 1:500 dilution of mouse anti-human YKL-40 antibody (QUIDEL, San Diego, CA) and 1:500 dilution of rabbit anti-MMP-1 antibody (Chemicon, Temecula, CA) or 1:500 dilution of rabbit anti-CD68 antibody (Santa Cruz, Santa Cruz, CA) for 2 h, and then incubated with a 1:1,000 dilution of Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen) for 30 min at 37˚C. The cells were observed under a fluorescence microscope.

Purification of YKL-40. *S*VF cells were incubated with serum–free DMEM for 24 h in collagen gels. The CM of 300 ml was fractionated by a 10-ml Gelatin-Sepharose 4B (GE Healthcare, Uppsala, Sweden) column. The non-gelatin-bound fraction was then passed through a 5-ml Hitrap Heparin HP column (GE Healthcare) equilibrated with 0.1 M phosphate buffer (pH 7.4). The heparin-bound material was eluted sequentially with 0.1 M phosphate buffer (pH 7.4) containing 300 mM, 400 mM, 500 mM, and 1 M NaCl with an AKTAprime chromatography system (GE Healthcare). Fractions eluted by 400 mM NaCl were concentrated with Centriplus YM-3K (Millipore).

Measurement of collagenolytic activity. The assay was performed as directed by the protocol of the Type I Collagenase Activity Assay Kit (LIFE, Yamagata, Japan) with some modifications. Briefly, mixtures with final concentrations of 250 μg/ml of FITC-labeled type I collagen from calf skin, various concentrations of purified YKL-40, human MMP-1 active form, and ice cold 1 x neutralization buffer supplied with the kit in 200 μl were incubated at 37˚C for 1 h. Samples were denatured with 200 μl of

denature/stop solution supplied with the kit for 30 min at room temperature and then centrifuged at 6,000 x *g* for 15 min. The fluorescence of the supernatant was measured with a fluorescence spectrophotometer.

Collagen fibril formation assay. One mg of type I collagen (Nitta-Gelatin) was added to 0.5 ml of fibrillogenesis buffer (0.15 M NaCl, 60 mM $NaHCO_3$, 40 mM HEPES, pH 7.4). Purified YKL-40 or heat-denatured BSA was added to the sample, and the final volume was brought to 1.0 ml. This mixture was transferred to a cuvette and incubated at 37˚C. The process of fibrillogenesis was measured as turbidity change by monitoring the change in absorbance at 400 nm.

Measurement of matrix metalloproteinases (MMPs) in CM. Passaged preadipocytes were cultured for 24 h in serum-free DMEM with or without 50% macrophage-CM. After washing with PBS three times, the cells were cultured in fresh serum-free DMEM for 24 h and each CM was collected. Concentrations of MMP-1 and -2 in the CM were measured with MMP-1 and -2 Human Biotrak ELISA systems (GE Healthcare), respectively.

Statistical analysis. Correlation between YKL-40 and CD68 mRNA levels was assessed by Pearson correlations with SPSS14.0 for Windows (SPSS Inc., Chicago, IL). Statistical analysis was performed using a Student t-test to compare differences in values between the control and samples. A p value of less than 0.05 represented a statistically significant difference between compared data sets.

Results

Identification of YKL-40 as a major protein secreted from SVF cells

To investigate the proteins secreted from SVF cells, SDS-PAGE and proteomic analysis of each CM collected from SVF cells fractionated from human visceral and subcutaneous ATs were performed. In the CM of SVF cells from subcutaneous AT (sSVF), no specific bands except for that of albumin derived from calf serum were observed, whereas several bands were detected in the CM of SVF cells from visceral AT (vSVF) (Fig. 1A). To identify these proteins secreted from vSVF cells, visible bands were excised from gels and subjected to MALDI-TOF MS. Among the candidates, YKL-40 (40 kDa) was identified as the most abundant protein in CM of vSVF cells (Fig 1A). Real-time RT-PCR revealed that YKL-40 mRNA levels were higher in vSVF cells than in sSVF cells similar to the protein levels (Fig. 1B).

YKL-40 is secreted from macrophages among SVF cells

Because the primary SVF cells used for proteomic analysis included preadipocytes, macrophages, vascular endothelial cells, *etc*, identification of cells secreting YKL-40 among SVF cells was attempted. Immunostaining of vSVF cells revealed that the YKL-40-positive cells coincided with the cells expressing CD68, a macrophage marker (Fig. 1C). Furthermore, YKL-40 mRNA levels in each passaged preadipocyte derived from vSVF and sSVF cells were measured. YKL-40 mRNA levels in the preadipocytes from both visceral and subcutaneous AT were remarkably low compared with those in macrophages (Fig. 1D). These results suggest that YKL-40 was secreted from macrophages among SVF cells, but not preadipocytes. Furthermore, YKL-40

mRNA levels were positively correlated with CD68 mRNA levels in both visceral and subcutaneous ATs (Fig. 1E), suggesting that YKL-40 expression levels may reflect numbers of macrophages infiltrating into AT. To deny a possibility that YKL-40 expression in AT is specific in subjects with tumors, immunofluorescence staining of sections of visceral AT from a subject without tumors and inflammatory lesions was performed (see Supplemental Figure). The YKL-40-positive signals were observed in CD68-positive cells, indicating that YKL-40 expression in macrophages infiltrating into AT was independent of tumor or inflammatory lesions.

YKL-40 derived from SVF cells inhibits the collagenolytic activity of MMP-1

Type I collagen dominates the ECM of normal white ATs [20]. To investigate whether YKL-40 in SVF cells affects degradation of type I collagen, we attempted to purify YKL-40 secreted from vSVF cells. After SDS-PAGE and silver-staining, the single band was confirmed as YKL-40 by mass spectrometry (Fig. 2A). The purified YKL-40 inhibited dose-dependently the degradation of type I collagen by MMP-1 even in the presence of high concentration of MMP-1 (Fig. 2B). Furthermore, fibril formation assay revealed that YKL-40 increased the rate and the final quantity of collagen fibril formation (Fig. 2C).

Macrophage-CM increases MMP expressions in preadipocytes

To investigate under what circumstances MMP-1 expression is enhanced in AT, we measured MMP-1 mRNA levels in passaged preadipocytes stimulated by CM of macrophages differentiated from THP-1 or U937 cells. The addition of macrophage-CM significantly induced mRNA expression of MMP-1, but not MMP-2 in

preadipocytes derived from visceral AT (Fig. 3A). Furthermore, monocyte chemoattractant protein-1 (MCP-1) mRNA levels were also increased by macrophage-CM (Fig. 3A). The macrophage-CM induced MMP-1's secretion from preadipocytes, but not MMP-2's secretion in a similar pattern to the mRNA levels (Fig. 3B). These results suggest that soluble factors secreted by macrophages induced MMP-1's expression in preadipocytes.

Co-culture of preadipocytes and macrophages increases MMP-1 expression

Next, to investigate the expression of MMP-1 in AT accompanied by the infiltration of macrophages, we performed immunocytochemistry of co-cultured preadipocytes and macrophages. Immunostaining using anti-YKL-40 and anti-CD68 antibodies revealed that YKL-40 was exclusively expressed in CD-68-positive cells (Fig. 4A). Although little MMP-1 protein was observed in macrophages or preadipocytes in single cultures, the co-culture of preadipocytes and macrophages markedly increased MMP-1 protein expression in both YKL-40-positive and negative cells (Fig. 4B). These results suggested that interaction between preadipocytes and macrophages induced production and secretion of MMP-1 in both cells.

Discussion

We identified YKL-40 as a major protein secreted from vSVF cells. SDS-PAGE and real-time RT-PCR revealed that the secretion and the gene expression of YKL-40 were greater in vSVF than in sSVF. This might be caused by a difference in the numbers of macrophages among SVF cells from each AT, because the infiltration of macrophages into visceral AT is greater than that into subcutaneous AT [21]. Indeed, we showed that YKL-40 was expressed in macrophages among SVF cells and the mRNA levels in both visceral and subcutaneous ATs correlated with the CD68 mRNA levels, suggesting that YKL-40 expression levels in AT were dependent on the number of macrophages in AT. We could not rule out the possibility that the expression of YKL-40 was dependent on tumor status, because plasma levels of YKL-40 are known to be increased in various cancer patients [15,16]. However, immunohistochemical study of AT from a subject without tumors and inflammatory lesions at least revealed that macrophages in AT secrete YKL-40 in subjects without tumors.

Bigg *et al*. [19] reported that YKL-40 in bovine nasal cartilage and chondrocytes has three isoforms, a major and minor form from resorbing cartilage and a third species from chondrocytes. The chondrocyte-derived species prevents cleavage of type I collagen and stimulates the rate of fibril formation of type I collagen. On the other hand, the major form from cartilage had an inhibitory effect on the formation of type I collagen fibrils. These indicate that YKL-40 derived from different tissues may have different roles. We found that YKL-40 derived from human vSVF cells has the function of YKL-40 in human SVF cells is similar to that of the chondrocyte-derived species rather than the major form from cartilage in bovine.

The mRNA expressions of MMP-1 and MCP-1 in preadipocytes were enhanced by addition of macrophage-CM, suggesting that MMP-1 and MCP-1 expression in

preadipocytes is induced under condition that macrophage is activated by some inflammatory signals. MMPs are thought to be involved in the infiltrations of inflammatory cells such as macrophages by degradation of the ECM, and MCP-1 is known to affect the accumulation and function of macrophages and the expression has been found in SVF cells and increases in obese subjects [22]. Induction of MMP-1 and MCP-1 in preadipocytes may coordinately contribute to the infiltration of macrophages into AT by cleaving type I collagen of AT to facilitate the infiltration and by chemo-attracting macrophages into AT, respectively.

In vitro study revealed that YKL-40 blocked degradation of type I collagen dose-dependently, irrespective of the concentration of MMP-1. YKL-40 purified from vSVF cells inhibited the degradation of type I collagen by MMP-1 at a final concentration 100 ng/ml and more. The effective concentration of YKL-40 was regarded as physiological because most reports showed that the range of YKL-40 plasma levels is 25 - 200 ng/ml [15,17,18] and the local concentration of YKL-40 in AT is presumably higher than this. Although stoichiometric relation between YKL-40 levels and MMP-1 activity in the vicinity of AT-infiltrating macrophages *in vivo* remains unclear, degradation of type I collagen may be strongly inhibited at the location with high concentration of YKL-40 such as neighborhood of macrophages, even if interaction between macrophages and preadipocytes increases MMP-1 expression. Therefore, YKL-40 secreted from macrophages in AT may contribute to trap macrophages into AT by inhibition of type I collagenolysis by MMP-1.

Taken together, our results suggest the preliminary hypothesis about macrophage-infiltration into AT; macrophages activated by inflammatory signals induce MMP-1 and MCP-1 expression in preadipocytes to infiltrate macrophages into AT, and then YKL-40 in infiltrating macrophages reduces MMP-1 activity and accelerates the fibril formation at vicinity of macrophages to trap macrophages in AT.

To demonstrate this hypothesis, further investigations are necessary.

Acknowledgments

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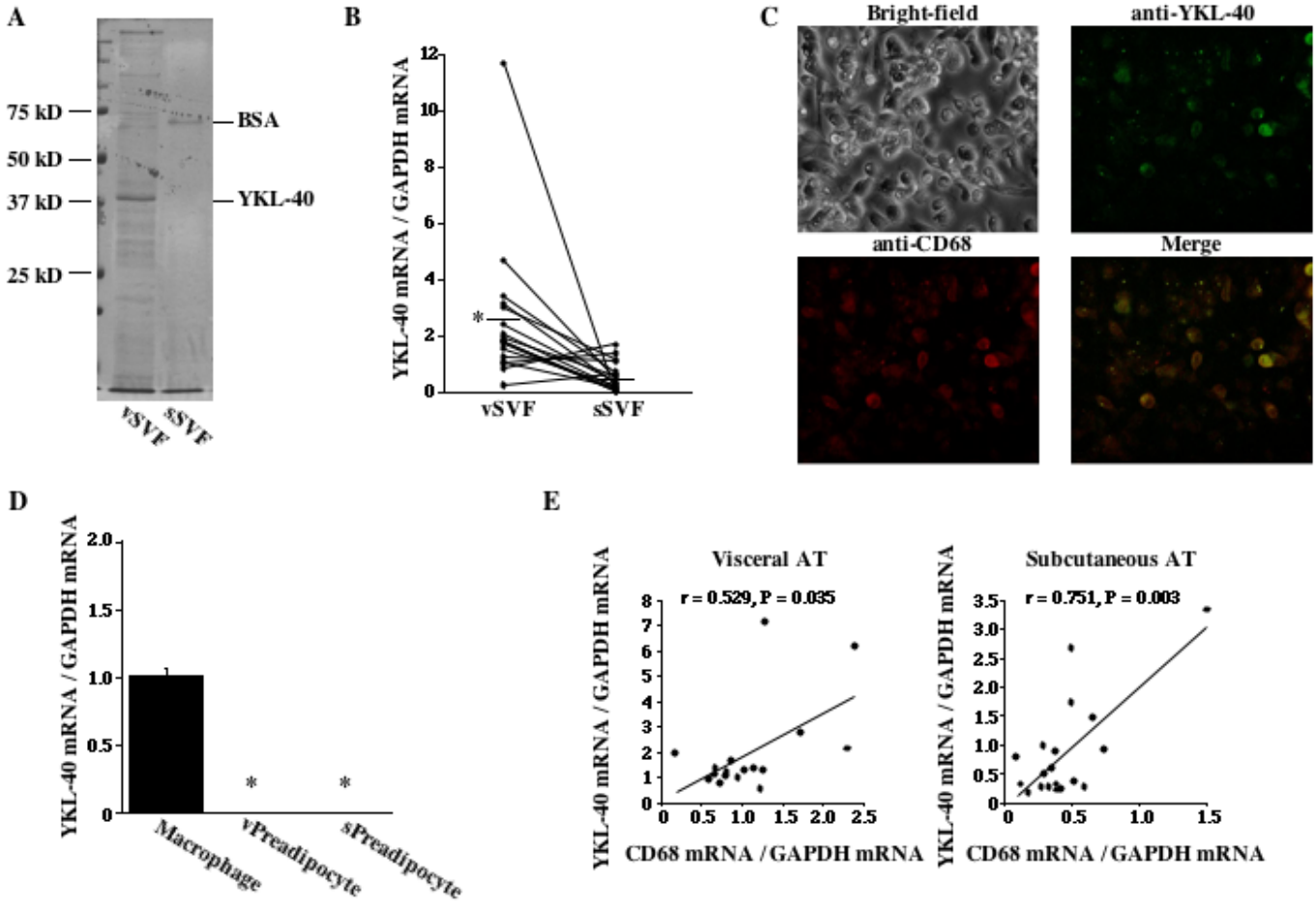
Figure legends

Fig. 1 YKL-40 secreted from macrophages among human SVF cells. (A) SDS-PAGE and CBB-staining of CM of SVF cells. YKL-40 was identified as the most remarkable band in CM of vSVF cells. (B) YKL-40 mRNA levels in paired vSVF and sSVF cells from 17 patients. The data are plotted with lines connecting the mRNA levels of vSVF and sSVF cells from the same participants. Mean values for overall group of each SVF cell are also indicated (horizontal bars). $*P < 0.05$. (C) Detection of cells expressing YKL-40 among vSVF cells by immunostaining with anti-YKL-40 (green) and anti-CD68 (red) antibodies. (D) YKL-40 mRNA levels in macrophages differentiated from THP-1 cells and respective passaged preadipocytes fractionated from visceral (vPreadipocyte) and subcutaneous (sPreadipocyte) AT. The data are the mean ± SEM for three independent experiments in triplicate (n=9). (E) Correlation between YKL-40 and CD68 mRNA levels in visceral or subcutaneous AT in 17 subjects.

Fig. 2 The effect of YKL-40 purified from vSVF on collagen degradation. (A) Silver staining of YKL-40 purified from CM of vSVF cells. (B) Dose-effect of MMP-1 on collagenolytic activity in the presence of various YKL-40 concentrations. The collagenolytic activity was presented as the percentage of fluorescent intensity derived from collagen degraded per hour. The data are the mean ± SEM for three independent experiments in triplicate (n=9). $*P < 0.05$. (C) Effect of YKL-40 on type I collagen fibril formation. Purified YKL-40 or heat-denatured BSA was added to type I collagen dissolved in fibril formation buffer and incubate at 37˚C during indicating time. The data are the mean ± SEM for three independent experiments in triplicate (n=9). $*P < 0.05$.

Fig. 3 MMPs expression in passaged preadipocytes derived from vSVF treated with macrophage-CM or inflammatory cytokines. (A) Effect of macrophage-CM on mRNA levels of MMP-1 and -2 in preadipocytes. Each mRNA level was measured in preadipocytes which were cultured for 24 h in serum-free DMEM (open column) or 50% CM of macrophages differentiated from THP-1 (gray column) and U937 (black column). (B) Effect of macrophage-CM on secretion of MMP-1 and -2 from preadipocytes. Incubation with serum-free DMEM and 50% CM of macrophages derived from THP-1 cells for 24 h is represented by an open column and a filled column, respectively. Each graph shown is representative of three independent experiments. Values are expressed as the mean ± SEM (n=3). $*P < 0.05$.

Fig. 4 MMP-1 expression in co-culture of preadipocytes derived from vSVF and macrophages. (A) Detection of YKL-40-expressing cells (green) in co-cultures of preadipocytes and macrophages using anti-CD68 antibody (red). YKL-40 expressed in only CD68 positive cells. (B) Immunocytochemistry using anti-YKL-40 (green) and anti-MMP-1 (red) antibodies in co-cultures of preadipocytes and macrophages, or in single-cultures of each cell type.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75 kD
50 kD
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BSA
YKL-40
vSVF
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B
YKL-40 mRNA / GAPDH mRNA
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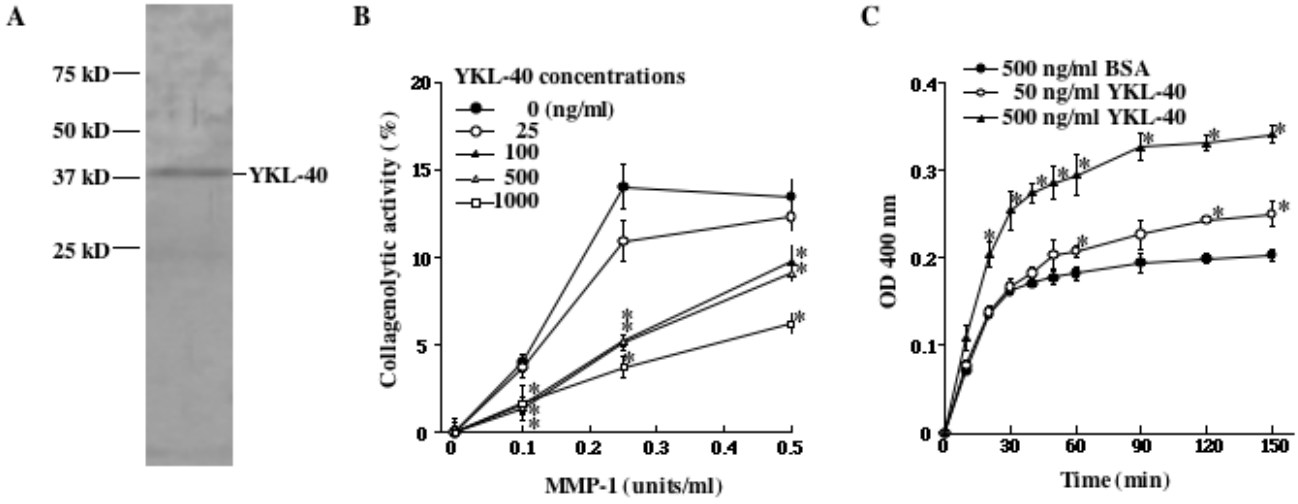
sSVF
C
Bright-field
anti-YKL-40
anti-CD68
Merge
D
YKL-40 mRNA / GAPDH mRNA
Macrophage
vPreadipocyte
sPreadipocyte
E
Visceral AT
r = 0.529, P = 0.035
Subcutaneous AT
r = 0.751, P = 0.003
YKL-40 mRNA / GAPDH mRNA
CD68 mRNA / GAPDH mRNA

Fig. 1


A
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YKL-40
B
YKL-40 concentrations
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Collagenolytic activity (%)
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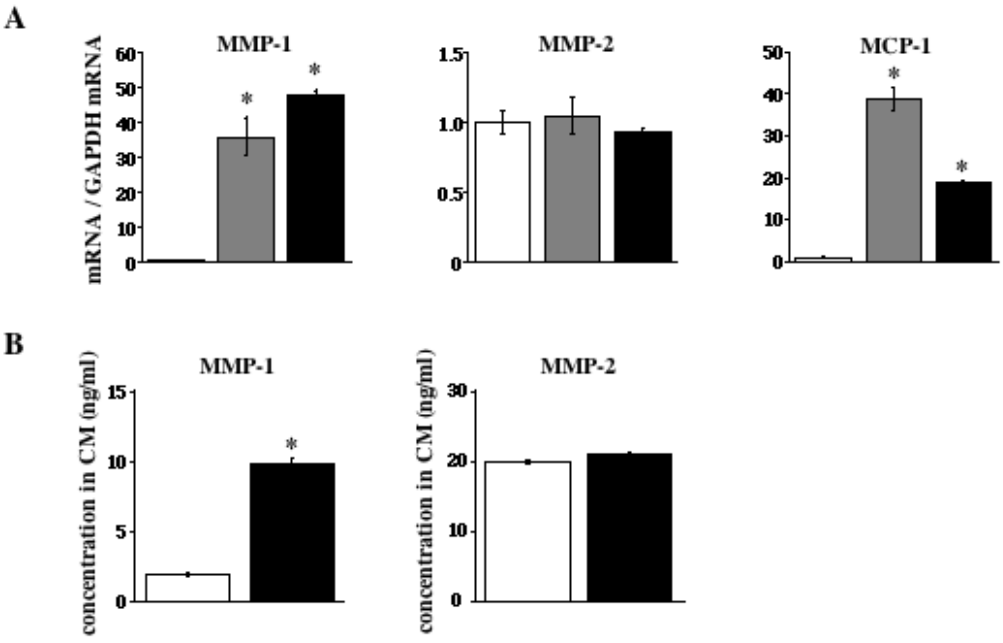
Time (min)

Fig. 2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MMP-2
MCP-1
mRNA / GAPDH mRNA
60
50
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1.5
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B
MMP-1
MMP-2
concentration in CM (ng/ml)
15
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5
0
30
20

Fig. 3

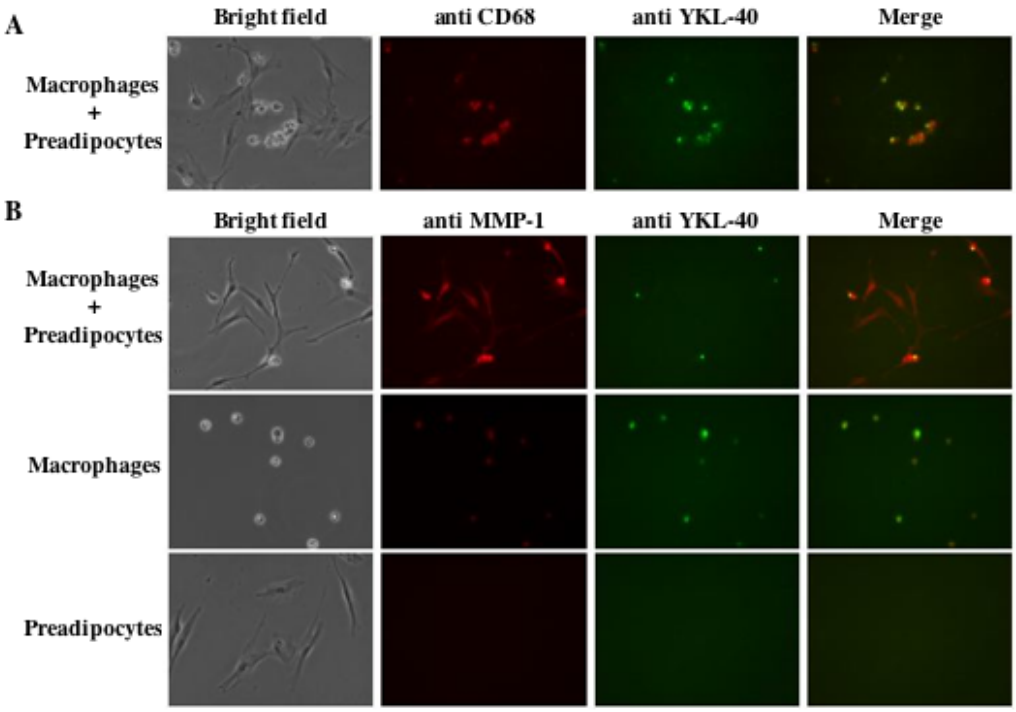
A
Bright field
anti CD68
anti YKL-40
Merge
Macrophages
+
Preadipocytes
B
Bright field
anti MMP-1
anti YKL-40
Merge
Macrophages
+
Preadipocytes
Macrophages
Preadipocytes

Fig. 4

Supplemental data

YKL-40 secreted from adipose tissue inhibits degradation of type I collagen

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Supplementary Table 1 Clinical data of obesity and cancer types of subjects who participated in this study.

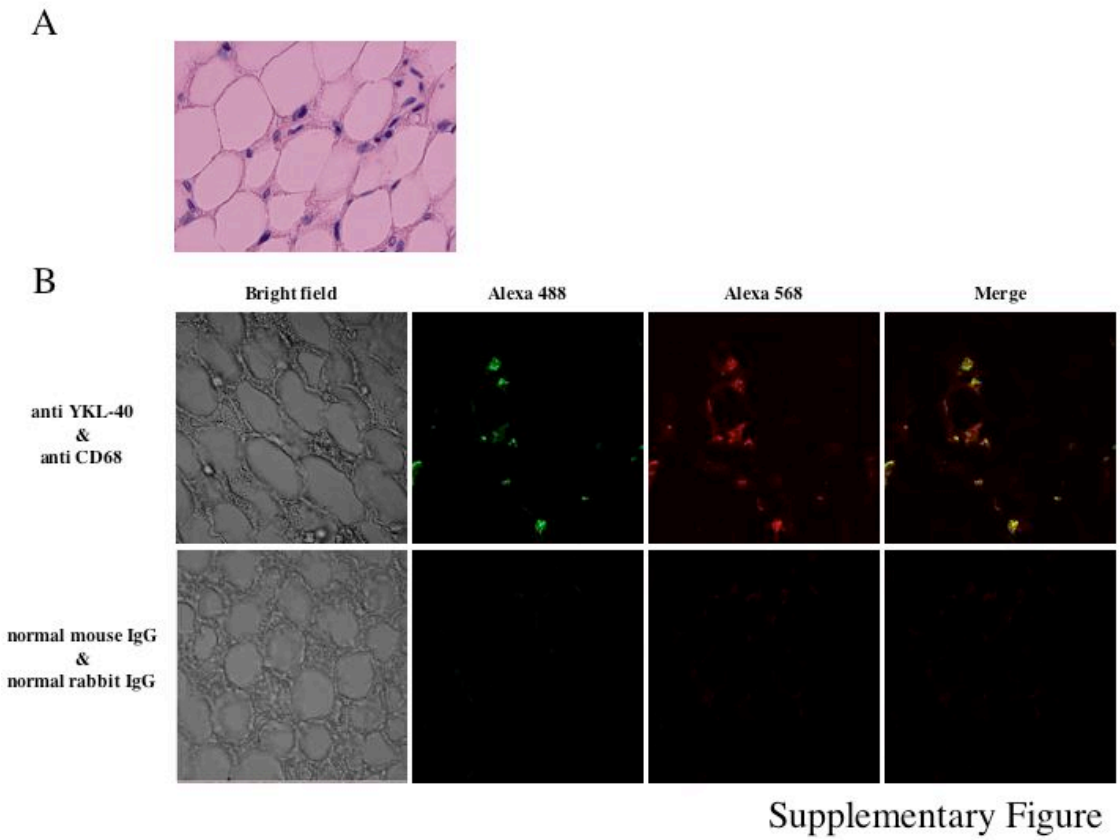
No.	sex	age	body mass index	visceral fat area (cm^2)	subcutaneous fat area (cm^2)	cancer type
1	female	70	18.2	47.3	80.2	inflammatory pseudotumor of the liver
2	male	74	25.0	169.2	163.6	gastric cancer
3	female	72	20.9	84.6	102.2	gallbladder cancer
4	female	56	19.1	36.5	84.6	gastric cancer
5	female	78	20.2	91.6	74.8	pancreatic cancer
6	male	60	19.5	67.4	87.6	cholangiocllular carcinoma
7	male	73	20.5	150.1	150.1	colon cancer
8	female	77	25.8	81.4	239	pancreatic ductal papillary adenocarcinoma
9	female	55	29.0	261.7	190.4	uterine corpus cancer
10	female	58	20.6	54.7	201.9	uterine cervical cancer
11	male	46	28.6	219.0	251.6	sigmoid colon cancer
12	male	77	22.1	120.7	117.0	sigmoid colon cancer
13	female	53	28.5	84.8	333.6	uterine corpus cancer
14	female	46	23.7	160.4	204.8	ovarian cancer
15	female	54	20.5	41.8	125.6	sigmoid colon cancer
16	male	80	23.6	183.5	127.9	gastric cancer
17	female	44	32.8	167.5	310.6	gastric cancer

Visceral and subcutaneous adipose tissue areas at the umbilical level in abdominal computed tomography were determined using commercially available software (Fat Scan; N2 System, Osaka, Japan). The subjects did not receive chemotherapy or radiotherapy before surgical operation.

Suplementary Table 2　Sequences of the PCR primers used for real-time RT-PCR in this study

PCR products		Primer Sequence
Human GAPDH	F	5'-GAAGGTGAAGGTCGGAGTC-3'
	R	5'-GAAGATGGTGATGGGATTTC-3'
Human YKL-40	F	5'-AAGGACACCATTTTGGCAAG-3'
	R	5'-AGTGGGCGCTCAAAGATAGA-3'
Human CD68	F	5'-GCTGGCTGTGCTTTTCTCG-3'
	R	5'-GTCACCGTGAAGGATGGCA-3'
Human MMP-1	F	5'-ATGCTGAAACCCTGAAGGTG-3'
	R	5'-CTGCTTGACCCTCAGAGACC-3'
Human MMP-2	F	5'-ATGACAGCTGCACCACTGAG-3'
	R	5'-ATTTGTTGCCCAGGAAAGTG-3'
Human MCP-1	F	5'-CCCCAGTCACCTGCTGTTAT-3'
	R	5'-TGGAATCCTGAACCCACTTC-3'

Supplementary Figure



Supplementary Figure. Detection of YKL-40 positive cells in visceral AT from a subject without tumors or inflammatory lesions. Paraffin sections of omental AT removed from a subject without tumors or inflammatory lesions were incubation overnight at 4˚C with 1:100 dilution of mouse anti-human YKL-40 antibody and 1:100 dilution of rabbit anti-CD68 antibody. After three washes in PBS, the sections were stained with Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG as the second antibodies for 30 min. The sections were visualized with a laser scanning confocal microscope (Carl Zeiss, Jena, Germany). Negative controls were used by omitting the primary antibody. Some sections were stained with hemotoxylin and eosin (H&E), and examined by light microscopy. (A) H&E staining of sections from visceral AT. (B) Immunostaining of sections of visceral AT with anti-YKL-40 (green) and anti-CD68 (red) antibodies.