

Serum Progranulin Concentrations May Be Associated With Macrophage Infiltration Into Omental Adipose Tissue

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OBJECTIVE—Progranulin is an important molecule in inflammatory response. Chronic inflammation is frequently associated with central obesity and associated disturbances; however, the role of circulating progranulin in human obesity, type 2 diabetes, and dyslipidemia is unknown.

RESEARCH DESIGN AND METHODS—For the measurement of progranulin serum concentrations, we developed an enzyme-linked immunosorbent assay (ELISA). Using this ELISA, we assessed circulating progranulin in a cross-sectional study of 209 subjects with a wide range of obesity, body fat distribution, insulin sensitivity, and glucose tolerance and in 60 individuals with normal (NGT) or impaired (IGT) glucose tolerance or type 2 diabetes before and after a 4-week physical training program. Progranulin mRNA and protein expression was measured in paired samples of omental and subcutaneous adipose tissue (adipocytes and cells of the stromal vascular fraction) from 55 lean or obese individuals. Measurement of Erk activation and chemotactic activity induced by progranulin *in vitro* was performed using THP-1–based cell migration assays.

RESULTS—Progranulin serum concentrations were significantly higher in individuals with type 2 diabetes compared with NGT and in obese subjects with predominant visceral fat accumulation. Circulating progranulin significantly correlates with BMI, macrophage infiltration in omental adipose tissue, C-reactive protein (CRP) serum concentrations, A1C values, and total cholesterol. Multivariable linear regression analyses revealed CRP levels as the strongest independent predictor of circulating progranulin. The extent of *in vitro* progranulin-mediated chemotaxis is similar to that of monocyte chemoattractant protein-1 but independent of α . Moreover, in type 2 diabetes, but not in IGT and NGT individuals, physical training for 4 weeks resulted in significantly decreased circulating progranulin levels.

CONCLUSIONS—Elevated progranulin serum concentrations are associated with visceral obesity, elevated plasma glucose, and dyslipidemia. We identified progranulin as a novel marker of chronic inflammation in obesity and type 2 diabetes that closely

reflects omental adipose tissue macrophage infiltration. Physical training significantly reduces elevated circulating progranulin in patients with type 2 diabetes. *Diabetes* 58:627–636, 2009

Immune response and metabolic regulation are highly integrated, and the proper function of each is dependent on the other (1). Chronic inflammation is frequently associated with central obesity and associated disturbances (2). In patients with obesity and type 2 diabetes, altered serum concentrations of inflammatory markers, including high-sensitivity C-reactive protein, tumor necrosis factor- α (TNF- α), fibrinogen, interleukin (IL)-10, adiponectin, and IL-6, have been frequently reported (2–5). However, the link between inflammation and central obesity is not fully understood.

Progranulin is a secreted protein with important functions in several processes, including immune response and embryonic development (6). Progranulin, also known as granulin, acrogranin, proepithelin, and PC cell–derived growth factor, is a 593–amino acid glycoprotein, the mRNA of which is expressed in many epithelial cells both *in vitro* and *in vivo*. The widespread occurrence of progranulin mRNA in cells from the hematopoietic system and in epithelia implies important functions in these tissues. Autosomal dominant mutations in the progranulin gene cause frontotemporal dementia (7,8), whereas overexpression of progranulin promotes the invasive progression of a range of tumors, including those of the breast and the brain (9). It has been shown that overexpression of progranulin renders breast cancer cells resistant to tamoxifen (10), suggesting that autocrine regulation of progranulin by cancer cells is critically involved in breast cancer tumorigenesis (11). It was also found that in murine transcutaneous puncture wounds, progranulin mRNA is expressed in the inflammatory infiltrate and is highly induced in dermal fibroblasts and endothelia after injury (12). When applied to a cutaneous wound, progranulin increased the accumulation of neutrophils, macrophages, blood vessels, and fibroblasts in the wound (13), suggesting that progranulin could function as a chemotactic protein for myeloid-origin cell types and an angiogenic factor. A recent study showed that progranulin is an inducible protein in response to hypoxia or acidosis (14). Taken together, progranulin is an important molecule in inflammatory response and could therefore be involved in chronic subclinical inflammation associated with human obesity and type 2 diabetes. However, the role of circulating progranulin in human central obesity and its associated comorbidities is unknown. We therefore developed

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TABLE 1
Anthropometric and metabolic characteristics of the study groups

	Normal glucose tolerant		Type 2 diabetes	
	Men	Women	Men	Women
<i>n</i>	12	18	48	76
Age (years)	61 ± 12	56 ± 15	62 ± 7	62 ± 8
Body weight (kg)	91 ± 20*	89 ± 23	103 ± 17†	90 ± 17
BMI (kg/m ²)	29 ± 3*†	33 ± 4	35 ± 6*	33 ± 7
Body fat (%)	22 ± 1*†	41 ± 8	30 ± 5†	41 ± 6
A1C (%)	5.5 ± 1.8*	5.4 ± 0.7*	6.6 ± 1.0	6.6 ± 1.0
Fasting plasma glucose (mmol/l)	5.3 ± 1.2*	5.2 ± 0.6*	7.5 ± 2.2	7.2 ± 2.0
Fasting plasma insulin (pmol/l)	38 ± 12*†	58 ± 11*	136 ± 253	145 ± 116
Free fatty acids (mmol/l)	0.45 ± 0.48*	0.48 ± 0.27*	0.66 ± 0.25	0.62 ± 0.26
Cholesterol (mmol/l)	4.3 ± 0.6*†	5.2 ± 0.8	5.0 ± 1.0	5.5 ± 1.1
HDL cholesterol (mmol/l)	1.3 ± 0.2*†	1.6 ± 0.2*	1.1 ± 0.2†	1.4 ± 0.3
LDL cholesterol (mmol/l)	2.7 ± 0.6*†	3.4 ± 0.3*	3.3 ± 1.0†	3.7 ± 0.9
Triglycerides (mmol/l)	1.4 ± 0.6*	1.5 ± 0.8*	2.0 ± 0.7	2.0 ± 1.2
Leptin	9.7 ± 7.7*†	25.3 ± 16.9	14.8 ± 11.0†	28.8 ± 18.9
Adiponectin	5.5 ± 2.3*†	8.6 ± 3.8	3.7 ± 2.4	6.5 ± 3.2
hsCRP (mg/dl)	0.22 ± 0.1*	0.29 ± 0.1*	0.34 ± 0.27†	0.45 ± 0.36

Data are means ± SD. **P* < 0.05 for patients with type 2 diabetes versus normal glucose tolerant individuals within each sex. †*P* < 0.05 for difference between men and women within the normal glucose tolerant or type 2 diabetes group.

an enzyme-linked immunosorbent assay (ELISA) for the measurement of human progranulin serum concentrations. To this end, a human progranulin-specific polyclonal antibody (PAb) was generated, which was then used to create a sandwich ELISA. Using this new progranulin ELISA, we sought to determine circulating progranulin in individuals with a wide range of obesity, body fat distribution, insulin sensitivity, and glucose tolerance. In addition, we assessed progranulin serum concentration before and after an intensive 4-week physical training program to test whether progranulin levels are regulated in response to training-associated improvements in body weight and insulin sensitivity. Adipose tissue has gained recognition as an important contributor to chronic systemic inflammation (15–17). Especially omental macrophage infiltration correlates with measures of central obesity and associated disturbances (17,18). Different adipokines, including monocyte chemoattractant protein-1 (MCP-1), have been shown to recruit monocytes into adipose tissue (19). We therefore tested the hypothesis that progranulin could facilitate monocyte recruitment into adipose tissue and that circulating progranulin reflects macrophage infiltration into omental adipose tissue.

RESEARCH DESIGN AND METHODS

We included three different cohorts with a total number of 269 individuals in our study of progranulin serum concentration, adipose tissue mRNA, and protein expression. In the first cohort (*n* = 154), we investigated progranulin serum concentrations in relation to measures of obesity and glucose metabolism in a cross-sectional study (cohort 1). In another cross-sectional study (cohort 2), we investigated progranulin mRNA and protein expression in paired omental and subcutaneous adipose tissue samples in addition to progranulin serum concentrations (*n* = 55). In a third study (cohort 3), we investigated circulating progranulin in response to a 4-week intensive exercise intervention in 60 individuals with different degrees of glucose tolerance. Individuals of all three cohorts fulfilled the following inclusion criteria: 1) Absence of any acute or chronic inflammatory disease as determined by a leukocyte count >7,000 Gpt/l, C-reactive protein (CRP) >5.0 mg/dl, or clinical signs of infection; 2) undetectable antibodies against glutamic acid decarboxylase; 3) no medical history of hypertension, i.e., systolic blood pressure was <140 mmHg and diastolic blood pressure was <85 mmHg; 4) no clinical evidence of either cardiovascular or peripheral artery disease; 5) no thyroid dysfunction; 6) no alcohol or drug abuse; and 7) no pregnancy. All study protocols have been approved by the ethics committee of the University of

Leipzig. All participants gave written informed consent before taking part in the study. The three cohorts had the following specific characteristics.

Cohort 1. A total of 154 Caucasian men (*n* = 60) and women (*n* = 98) have been consecutively recruited in the context of a study on insulin resistance at the Department of Medicine, University of Leipzig, to represent a wide range of obesity, insulin sensitivity, and glucose tolerance. The age ranged from 25 to 79 years and BMI from 20.2 to 61.5 kg/m². The study included 124 patients with type 2 diabetes and 30 normal glucose tolerant (NGT) control subjects (Table 1).

Cohort 2. Adipose tissue progranulin mRNA and protein expression was investigated in 55 previously described donors of paired omental and subcutaneous adipose tissue samples (20), who underwent open abdominal surgery for cholecystectomy, weight reduction surgery, abdominal injuries, or explorative laparotomy. All subjects had a stable weight, defined as the absence of fluctuations of >2% of body weight for at least 3 months before surgery. Adipose tissue was either analyzed as a whole (immediately frozen in liquid nitrogen after explantation) or after separation into isolated adipocytes and cells of the stromal vascular fraction (SVF) using previously described methods (21). Histological analyses and measurement of macrophage count in adipose tissue was performed as previously described (18). In brief, adipose tissue samples were fixed at room temperature in 4% formaldehyde and embedded in paraffin. Five-micrometer sections were mounted on glass slides, deparaffinized in xylol, and stained for CD68 using anti-CD68 monoclonal mouse antihuman antibody (clone PGM1 M0876, dilution 1:100; Dako, Glostrup, Denmark) or stained for human progranulin using the novel developed human polyclonal anti-progranulin antibody (AG 101; AdipoGen, Seoul, Korea) with standard immunostaining methods. Macrophages were identified in the adipose parenchyma when cytoplasmic staining for CD68 was present along with an identifiable mononuclear nucleus and presented as the number per 100 adipocytes (percent macrophages).

Cohort 3. Sixty subjects were divided into groups of NGT (*n* = 20; 9 men, 11 women), impaired glucose tolerance (IGT, *n* = 20; 9 men, 11 women), and type 2 diabetes (*n* = 20; 11 men, 9 women) on the basis of a 75-g oral glucose tolerance test (OGTT) according to the American Diabetes Association criteria (22). Subjects with NGT were defined by a fasting plasma glucose <6.0 mmol/l and a 120-min plasma glucose <7.8 mmol/l. Subjects with IGT were defined by a fasting plasma glucose <6.0 mmol/l, a 120-min plasma glucose >7.8 mmol/l and <11.1 mmol/l. Subjects with type 2 diabetes were defined by a fasting plasma glucose >7.0 mmol/l and/or a 120-min OGTT glucose >11.1 mmol/l.

These 60 individuals were enrolled in 60 min of supervised physical training sessions 3 days per week as described previously (4). In brief, each training session included 20 min of biking or running, 20 min of swimming, and 20 min of warming up/cooling down periods. All subjects completed a graded bicycle-ergometer test to volitional exhaustion and had maximal oxygen uptake (*V*_{O_{2max}) measured with an automated open circuit gas analysis system at baseline and after 4 weeks of training. The highest oxygen uptake/min reached was defined as the *V*_{O_{2max}, and subjects subsequently}}

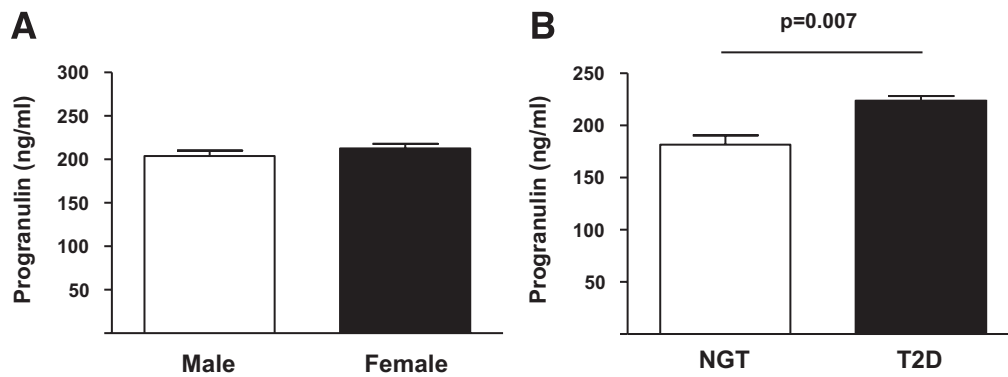


FIG. 1. Progranulin serum concentrations in NGT individuals and patients with type 2 diabetes (T2D). Circulating progranulin in men ($n = 12$) and women ($n = 18$) with NGT (A) and in individuals with either type 2 diabetes ($n = 124$) or NGT ($n = 30$) (B). Data are means \pm SE.

trained at their individual submaximal heart rate defined as 70–80% of the individual maximal heart rate during the bicycle-ergometer test. At baseline and after 4 weeks of training (48 h after the last training session), blood samples were obtained in the fasting state and measurements of anthropometric parameters were performed. We further assessed circulating progranulin before, immediately after, and after 24 and 48 h of an acute exercise circle training to exhaustion for 60 min in 20 healthy volunteers.

Measurement of body fat content, glucose metabolism, and insulin sensitivity. All baseline blood samples were collected between 8:00 and 10:00 A.M. after an overnight fast. Plasma insulin was measured with an enzyme immunometric assay for the IMMULITE automated analyzer (Diagnostic Products, Los Angeles, CA). Serum high-sensitive CRP was measured by immunonephelometry (Dade-Behring, Milan, Italy). Serum adiponectin and IL-6 were measured as previously described (4). Serum MCP-1 concentrations were measured by immunoassay system (Quantikine human MCP-1 Immunoassay; R&D Systems, Minneapolis, MN). BMI was calculated as weight divided by squared height. Hip circumference was measured over the buttocks; waist circumference was measured at the midpoint between the lower ribs and iliac crest. Percentage body fat was measured by dual X-ray absorptiometry. In addition, abdominal visceral and subcutaneous fat areas were calculated using computed tomography (CT) scans at the level of L4–L5 in the cohort of paired visceral and subcutaneous adipose tissue donors. Based on a previous report (23), visceral obesity was defined as >0.4 ratio of visceral to subcutaneous fat area as determined by CT scan. Three days before the OGTT, patients documented a high-carbohydrate diet in diet protocols. The OGTT was performed after an overnight fast with 75-g standardized glucose solution (Glucodex Solution 75 g; Merieux, Montreal, Canada). Venous blood samples were taken at 0, 60, and 120 min for measurements of plasma glucose concentrations. Insulin sensitivity was assessed with the euglycemic-hyperinsulinemic clamp method (24,25).

Progranulin ELISA development and measurement of progranulin serum concentrations. A cDNA sequence encoding the original signal peptide sequence and the mature peptide of human progranulin was amplified and digested with appropriate restriction enzymes and then cloned into both pAGCF (AdipoGen, Seoul, Korea). A FLAG tag was incorporated at the mature human progranulin peptide. pAGCF is an in-house eukaryotic vector whose expression is driven by the cytomegalovirus early promoter. The tagged protein was expressed in a human embryonic kidney cell line, HEK-293, and purified from conditioned media through anti-FLAG Sepharose column. Endotoxin was removed through two consecutive column chromatography using Detoxigel (Pierce, Rockford, IL). PAb (AG 101) was then produced by immunizing with recombinant FLAG-tagged human progranulin according to general protocols. Immunoglobulin fractions were prepared from serum and then biotinylated. FLAG-tagged progranulin was used for ELISA standard at a variety of dilutions. A sandwich ELISA format was designed with the use of a pair of PAb and biotinylated PAb. A total of 100 μ l of the human serum in 1:200 dilutions was applied to each well, which has been coated by 5 μ g/ml human progranulin-specific PAb and was incubated at 37°C for 1 h followed by washing three times with PBS with 0.05% Tween-20 (PBST). Per well, 100 μ l biotinylated PAb at 5 mg/ml was added. The secondary antibody reaction was performed at 37°C for 1 h followed by washing three times with PBST. Colorimetric reaction was conducted for 20 min with the use of horseradish peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA) diluted 1:1,000 in PBS and 2,2'-azino-bis(2-ethylbenzothiazoline-6-sulfonic acid)

(Pierce) as substrate. The optical density was measured at 450 nm. Its sensitivity was 32 pg/ml. Whereas the degree of precision of the ELISA system in terms of coefficient of variance (%) of intra-assay was between 3 and 6.9% (supplementary Table 1, available in an online appendix at <http://dx.doi.org/10.2337/db08-1147>), that of interassays was between 4.7 and 7.3% (supplementary Table 2, available in the online appendix). Spike recovery (supplementary Table 3, available in the online appendix) and linearity (supplementary Table 4, available in the online appendix) were in a range of 89–103 and 93–102%, respectively. Specificity was determined using human adiponectin, retinol binding protein 4, visfatin, leptin, plasminogen activator inhibitor-1 (PAI-1), TNF- α , resistin-like molecule- β , fatty acid binding protein 4, angiopoietin-like protein 3 (ANGPTL3), ANGPTL6, glutathione peroxidase 3, clusterin, resistin, mouse visfatin, rat visfatin, and mouse resistin (supplementary Table 5, available in the online appendix).

Progranulin mRNA and protein expression. Human *progranulin* mRNA expression was measured by quantitative real-time RT-PCR in a fluorescent temperature cyclers using the TaqMan assay, and fluorescence was detected on an ABI PRISM 7000 sequence detector (Applied Biosystems, Darmstadt, Germany). Total RNA was isolated from paired subcutaneous and visceral adipose tissue samples using TRIzol (Life Technologies, Grand Island, NY), and 1 μ g RNA was reverse transcribed with standard reagents (Life Technologies). From each RT-PCR, 2 μ l was amplified in a 26- μ l PCR using the Brilliant SYBR Green QPCR Core Reagent kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. Samples were incubated in the ABI PRISM 7000 sequence detector for an initial denaturation at 95°C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. The following primers were used: human progranulin, 5'-gaaggctcgatctgagaga-3' (sense) and 5'-ctcaaggctggctccaa-3' (antisense). SYBR Green I fluorescence emissions were monitored after each cycle. Human progranulin mRNA expression was calculated relative to the mRNA expression of *18S rRNA*, determined by a premixed assay on demand for human *18S rRNA* (PE Biosystems). Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 68°C and heating slowly to 95°C with measurement of fluorescence) at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis. Progranulin protein expression in visceral and subcutaneous adipose tissue and in isolated adipocytes and SVF was measured in a representative subgroup of 30 subjects by Western blot analysis using the novel generated human progranulin-specific PAb (AG 101). Three blots of the samples were scanned using a Molecular Dynamics Storm PhosphorImager and quantified with ImageQuant software.

Detection of Erk activation in cells treated with recombinant human progranulin. To examine the signals of phospho-p44/42 mitogen-activated protein kinase (MAPK) and p44/42 MAPK, MCF10A, a human breast epithelial cell line, and THP-1, a human monocytic leukemic cell line, were serum-starved for 24 h and treated with 500 ng/ml or 100 ng/ml recombinant human progranulin, respectively. Cell lysates were prepared with radioimmunoprecipitation assay buffer. Equal amounts of protein were subjected to Western blot analyses with phosphorylation-specific Erk and pan-Erk antibodies (Cell Signaling Technology, Danvers, MA).

Measurement of chemotactic activity of human progranulin using THP-1 cells. Cell migration of THP-1 monocytic leukemia cells was carried out in 24-well transwell polystyrene membrane with 8- μ m pore size. 2×10^5 THP-1 cells were added to the upper chamber. A total of 100 ng/ml hProgranulin-FLAG was diluted in RPMI 1640 supplemented with 0.5% fetal bovine serum (FBS) and was then added to the low chamber. Together with THP-1

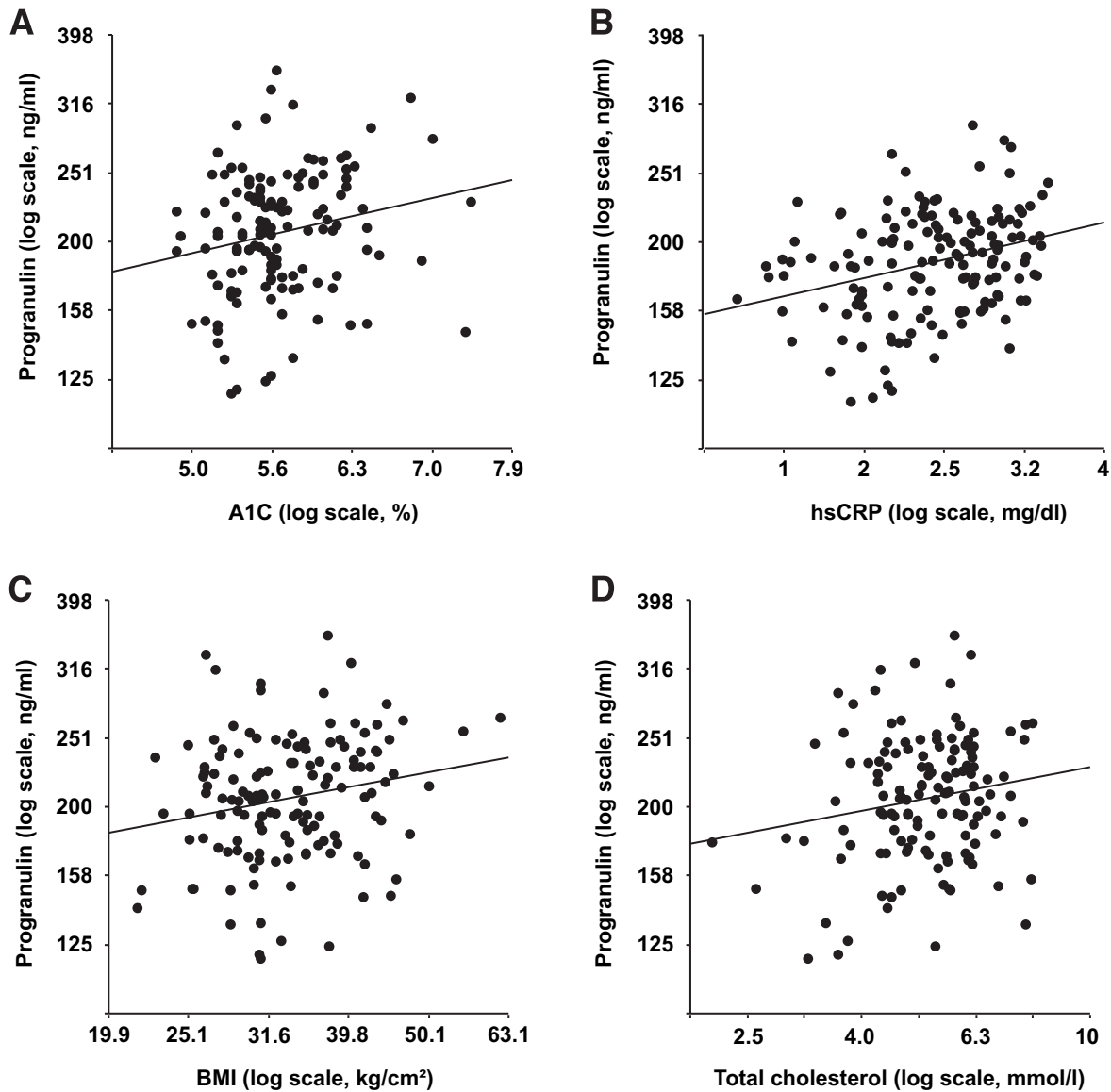


FIG. 2. Relationship between progranulin serum concentrations and anthropometric and metabolic parameters. Correlation between circulating progranulin and A1C ($r^2 = 0.25$, $P < 0.01$) (A), circulating CRP ($r^2 = 0.4$, $P < 0.001$) (B), BMI ($r^2 = 0.2$, $P < 0.05$) (C), and total cholesterol serum concentration ($r^2 = 0.2$, $P < 0.05$) (D). Data are log transformed to achieve normal distribution. hsCRP, high-sensitivity C-reactive protein.

cells (2×10^5 cells per well), 0, 10, 50, 100, and 250 ng/ml hProgranulin were added into the upper chamber. As a control, human progranulin was boiled for 10 min. After 16-h incubation at 37°C in a 5% CO₂ humidified atmosphere, migrated cells in the lower chamber were counted. Migrated cells in two separate fields per well from duplicate wells were enumerated on a hemacytometer by means of light microscopy. For a modified checkerboard analysis, 100 ng/ml human progranulin was placed into the low chamber, and a mixture of 2×10^5 THP-1 cells and varied concentrations of human progranulin (0, 10, 50, 100, and 250 ng/ml) was added into upper chambers. As control, human MCP-1 was used for chemotaxis experiments. For inhibitory chemotaxis assays, THP-1 cells were pretreated at 37°C for 1 h with 1 µg/ml pertussis toxin (PTX) or 20 µmol/l PD98059, washed, and placed into upper chambers. **Statistical analyses.** Data are shown as means ± SE unless stated otherwise. Before statistical analysis, non-normally distributed parameters were logarithmically transformed to approximate a normal distribution. The following statistical tests were used: paired Student's *t* test, χ square test, and Pearson's simple correlation. Linear relationships were assessed by least square regression analysis. Statistical analysis was performed using SPSS version 12.0 (Chicago, IL). *P* values <0.05 were considered to be statistically significant.

RESULTS

Progranulin serum concentrations in obesity and type 2 diabetes. Anthropometric and metabolic characteristics of 154 individuals in the first cross-sectional study

are summarized in Table 1. The current ELISA system was made possible by human progranulin-specific PAb. No cross-reactivity was observed with the use of a number of classical or new adipokines (supplementary Table 5). The progranulin ELISA ran according to the supplementary methods (supplementary tables, available in the online appendix). Circulating progranulin was not different between men and women with NGT (Fig. 1A) and type 2 diabetes (data not shown). Progranulin levels were 1.4-fold higher in individuals with type 2 diabetes compared with NGT (Fig. 1B) ($P = 0.007$). In these patients with a wide range of BMI and glucose tolerance, we found significant relationships between circulating progranulin and parameters of central obesity and its associated comorbidities. Univariate regression analyses revealed significant correlations between progranulin serum concentrations and A1C, CRP serum concentrations, BMI, and total cholesterol serum concentrations (Fig. 2). There was no correlation between serum progranulin concentration and circulating IL-6 ($r = 0.08$, $P = 0.7$) and adiponectin ($r = -0.14$, $P = 0.3$), whereas circulating progranulin and

TABLE 2
Multivariable linear regression analysis of different parameters as predictors of progranulin serum concentration ($n = 154$)

	Progranulin serum concentration	
	β -Coefficient	P value
Model 1		
Age	0.084	0.306
Sex	0.078	0.341
Model 2		
Age	0.143	0.076
Sex	0.039	0.624
hsCRP	0.308	<0.0001
Model 3		
Age	0.125	0.142
Sex	0.020	0.817
hsCRP	0.294	0.001
A1C	0.144	0.089
Model 4		
Age	0.130	0.130
Sex	0.030	0.728
hsCRP	0.261	0.006
A1C	0.145	0.091
BMI	0.082	0.381

Significant correlations are shown in bold.

MCP-1 significantly correlate ($r = 0.24$, $P = 0.02$). Multivariable linear regression models revealed that circulating CRP is the strongest predictor of progranulin serum concentration (Table 2). Associations between circulating progranulin and BMI or A1C are not independent of the significant relationship between circulating progranulin and CRP (Table 2).

Circulating progranulin correlates with macrophage infiltration into adipose tissue. We investigated progranulin mRNA and protein expression in visceral and subcutaneous adipose tissue in parallel with progranulin serum concentrations in a group of lean and obese individuals, which have been classified into lean or predominantly subcutaneously or viscerally obese on the basis of abdominal visceral and subcutaneous fat area measurements using CT scans at the level of L4–L5 as previously described (23,25). Progranulin levels were not significantly different between subcutaneous obese and lean individuals, whereas patients with a predominantly visceral fat distribution had significantly higher progranulin serum levels ($P = 0.01$) (Fig. 3A). There is a significant correlation between circulating progranulin and visceral fat area as measured by CT scan ($r = 0.42$, $P < 0.001$). In this cohort, we further investigated the relationship between circulating progranulin and macrophage count in subcutaneous and omental adipose tissue. Macrophage number was significantly higher in visceral than in subcutaneous fat (18). We found a strong correlation between circulating progranulin and both subcutaneous ($r = 0.4$, $P = 0.001$) and omental (Fig. 3B) macrophage number in adipose tissue. These relationships remained significant even on adjustment for age, sex, BMI, total body fat mass, and circulating CRP. Progranulin mRNA and protein expression was significantly higher in visceral compared with subcutaneous fat (Fig. 3C and D). We further sought to dissect the contribution of adipocytes and cells of the SVF on progranulin expression in adipose tissue. Immunostaining of adipose tissue sections using the new anti-progranulin antibody demonstrates that progranulin protein is detectable in vessels and cytoplasm of adipocytes and

cells of the SVF (Fig. 3E). Expression analyses revealed that both isolated adipocytes and cells of the SVF contribute to progranulin mRNA expression adipose tissue (Fig. 3F). These results have been confirmed at the protein level (data not shown).

Visceral progranulin mRNA expression significantly correlates with circulating progranulin, CRP, and MCP-1; visceral fat area; BMI; percent body fat; macrophage number in visceral fat; HDL cholesterol; and glucose infusion rate during the steady state of an euglycemic-hyperinsulinemic clamp, whereas subcutaneous progranulin mRNA only correlates with CRP and subcutaneous macrophage number (Table 3).

Progranulin activates Erk in vitro. Overexpression of progranulin has been shown to activate stress-activated kinase Erk gene in different tumor cell types (10) and progranulin seems to act on myeloid cells (13). However, its agonistic potential in vitro has not been substantiated. We therefore tested whether our recombinant human progranulin may activate Erk in the human breast cancer cell line MCF10A and in THP-1. As early as after 10 min of progranulin treatment, we found significantly increased Erk phosphorylation (Fig. 4A and B) with a sustained increase up to 60 min, confirming that progranulin is an Erk activator when used as a ligand in vitro.

Progranulin induces chemotactic activity in vitro in a $G\alpha$ -independent and an Erk-dependent manner. Infiltration of macrophages into omentum is a hallmark of human visceral obesity. To investigate the potential causal role of progranulin on monocyte recruitment into adipose tissue in more detail, we tested whether progranulin promotes cell migration in vitro. To test the hypothesis that progranulin is a monocyte chemoattractant molecule, THP-1 cell-based migration assays have been performed. A typical bell-shaped curve cell migration pattern was induced by different concentrations of human recombinant progranulin with a maximal migration index at a concentration of 100 ng/ml (Fig. 4C). To test whether this migration pattern is chemotactic or chemokinetic, a modified checkerboard analysis was performed. A significant decrease in migrated THP-1 cells treated with progranulin placed in upper chambers strongly suggests progranulin as a chemotactic factor for human macrophages (Fig. 4D). We further compared chemotactic activity of progranulin and MCP-1 using the THP-1 cell-based migration assays. At concentrations of 100 ng/ml, progranulin and MCP-1 exerted indistinguishable chemotactic activity (Fig. 4E). THP-1 cells were pretreated with PTX, which uncouples endogenous G protein α ($G\alpha$) subunits from G-protein-coupled receptors. These experiments revealed that progranulin-mediated chemotaxis of human macrophage-like cells is $G\alpha$ independent based on the observation that PTX did not affect cell migration in progranulin-treated cells, whereas MCP-1-mediated chemotaxis is $G\alpha$ dependent (Fig. 4E). Because progranulin was able to activate Erk in THP-1 cells, we tested whether pharmacological inhibition of Erk affects chemotaxis. Inhibition of Erk by the specific Erk inhibitor PD98059 significantly attenuated the progranulin-mediated chemotaxis, strongly suggesting that progranulin-mediated chemotaxis is Erk dependent (Fig. 4F).

Circulating progranulin in response to 4 weeks of intensive exercise training. Sixty Caucasian men and women completed a 4-week training program and were studied after being divided into subjects with NGT ($n = 20$), IGT ($n = 20$), or type 2 diabetes ($n = 20$) as previously

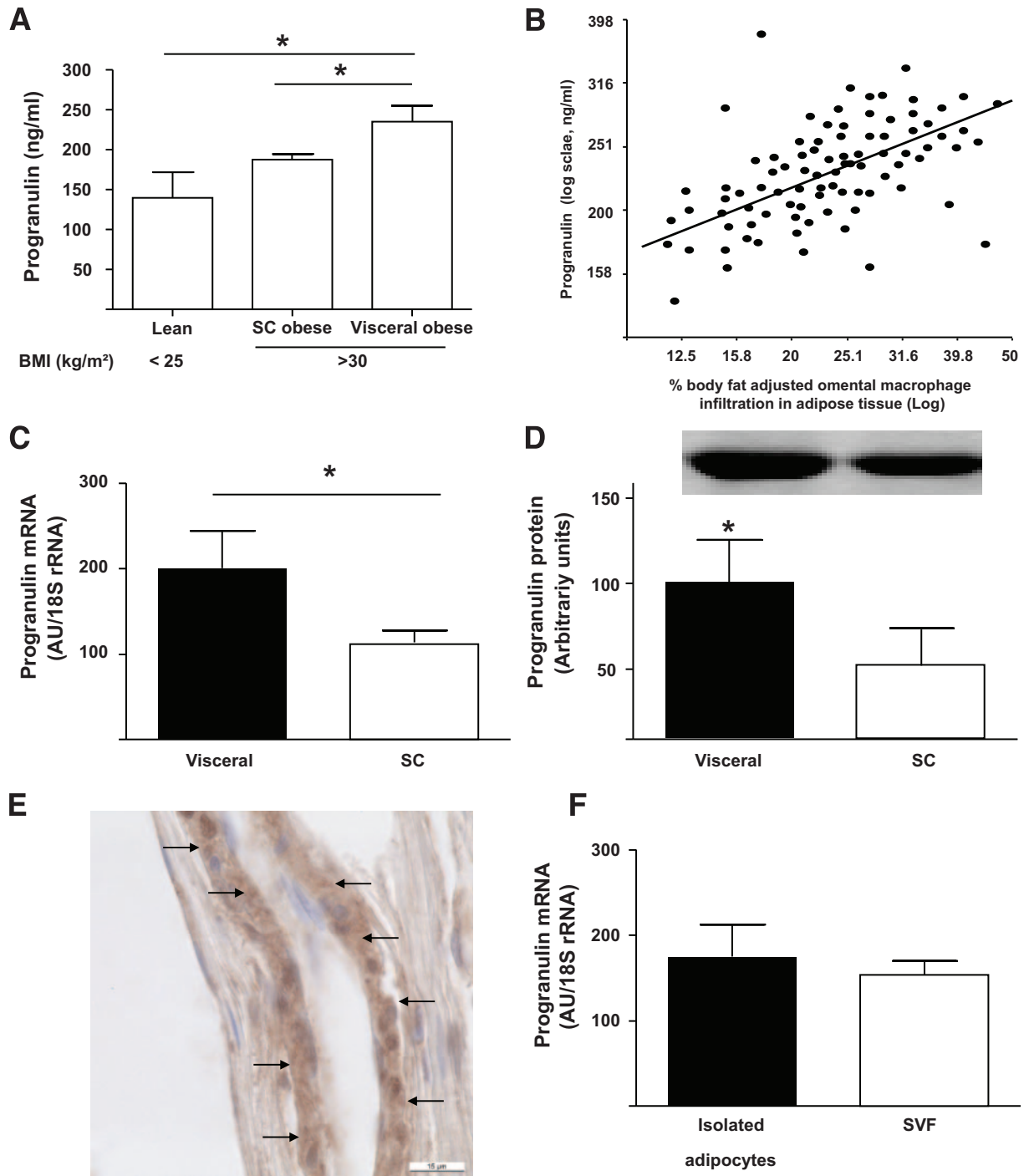


FIG. 3. Progranulin serum concentrations in visceral obesity. **A:** Circulating progranulin in lean, visceral obese, and subcutaneous (SC) obese individuals with NGT. Abdominal visceral and subcutaneous fat areas were calculated using computed tomography (CT) scans at the level of L4–L5 in the cohort of paired visceral and subcutaneous adipose tissue donors. Visceral obesity was defined as >0.4 ratio of visceral to subcutaneous fat area as determined by CT scan. Data are means \pm SE. * $P < 0.05$ between the groups. **B:** Correlation between circulating progranulin and macrophage infiltration into omental adipose tissue ($r^2 = 0.5$, $P < 0.001$). Data are log transformed to achieve normal distribution. Progranulin mRNA (**C**) and protein (**D**) expression in visceral and subcutaneous (SC) adipose tissue is shown. A representative Western blot is shown. **E:** Paraffin section of visceral adipose tissue of a normal glucose control individual. Immunostaining with a polyclonal anti-progranulin antibody. Arrows indicate progranulin-positive cytosol of adipocytes and cells of the stroma vascular fraction. **F:** Progranulin mRNA expression in isolated adipocytes and SVF. AU, arbitrary units. (Please see <http://dx.doi.org/10.2337/db08-1147> for a high-quality digital representation of this figure.)

described (4). The training effect was confirmed by a significant improvement in Vo_{2max} in all groups. As determined by matched paired *t* test ($P < 0.05$), all subjects had a significant increase in Vo_{2max} after the training period. Four weeks of physical training resulted in significant decreases in BMI, WHR, and percent body fat in all glucose

tolerance groups, and insulin sensitivity significantly improved in the IGT and type 2 diabetes groups (4).

Progranulin serum concentration significantly decreased by ~20% in type 2 diabetic subjects in response to the 4-week training program (Fig. 5A). There was a significant correlation between change in progranulin serum

TABLE 3

Univariate correlations between progranulin mRNA expression in visceral and subcutaneous adipose tissue and measures of obesity, insulin sensitivity, and parameters of inflammation ($n = 55$)

	<i>r</i>	<i>P</i> value	Adjusted for BMI	
			<i>r</i>	<i>P</i> value
Visceral progranulin mRNA				
Serum progranulin	0.27	<0.01	0.17	0.03
CRP	0.25	<0.01	0.18	0.02
MCP-1	0.18	0.03	0.06	0.6
Visceral fat area	0.46	<0.001	0.28	<0.01
BMI	0.41	<0.01	—	—
Body fat (%)	0.39	<0.01	0.04	0.7
Visceral macrophage				
number	0.62	<0.001	0.45	<0.001
HDL cholesterol	−0.35	<0.001	−0.12	0.1
GIR	−0.31	<0.01	−0.11	0.1
Subcutaneous progranulin mRNA				
Subcutaneous				
macrophage number	0.6	<0.001	0.38	<0.001
CRP	0.34	<0.001	0.22	0.01

GIR, glucose infusion rate during the steady state of a euglycemic-hyperinsulinemic clamp.

concentrations and change in CRP ($r^2 = 0.3$, $P = 0.007$) after the training program in all groups and in the entire cohort. Multivariable linear regression analysis further revealed that reduced CRP is a significant predictor of decreased progranulin serum concentration after training. In 20 healthy volunteers, we found significantly increased progranulin serum concentrations immediately after 60-min exercise circle training to exhaustion. Elevated progranulin serum concentrations normalized 24 h after the acute exercise (Fig. 5B).

DISCUSSION

We report here for the first time a strong association of elevated progranulin serum concentrations with type 2 diabetes, visceral fat accumulation, and increased macrophage infiltration into adipose tissue. Circulating progranulin levels correlate with markers of inflammation (CRP), chronic hyperglycemia (A1C), and predominantly visceral fat accumulation. Moreover, in an intervention study, we could demonstrate that elevated progranulin serum concentrations in patients with type 2 diabetes can be decreased by physical training.

Chronic inflammation and elevated (CRP, IL-6, TNF- α , and fibrinogen among others) or decreased (adiponectin, IL-10, and others) inflammatory markers are frequently associated with central obesity and associated disturbances (2–5). Increased amount of adipose tissue could contribute to altered serum profiles of inflammatory markers. Visceral fat depot-specific secretion of adipokines and immune cell-derived cytokines may at least in part explain the frequently reported association between central obesity and chronic inflammation (5,26,27). In this context, progranulin caught our attention, because it is a secreted protein with important functions in modulation of inflammatory events. In addition, the role of progranulin in obesity and type 2 diabetes has not been investigated.

Progranulin is a multifunctional protein that has gained attention in the neuroscience field, because of the discov-

ery that frontotemporal dementia is associated with progranulin mutations in some patients (28,29). The clinical phenotype of progranulin-null mutations includes behavioral abnormalities, language disorders, and Parkinson's disease (9). There is no systematic investigation of the metabolic consequences of mutations in the progranulin gene. The physiological function of progranulin is complex, with the full-length form of the protein having trophic and anti-inflammatory activity, whereas proteolytic cleavage generates granulin peptides that promote inflammatory activity (9). In the periphery, progranulin promotes wound healing by increasing the accumulation of neutrophils, macrophages, and other cells in wounds (13). We therefore hypothesized that progranulin plays a role in the association between obesity, type 2 diabetes, and inflammatory response and in macrophage accumulation into adipose tissue in patients with obesity.

We developed an ELISA for the measurement of human serum progranulin concentrations. Progranulin serum concentrations are not different in men and women and do not seem to be age dependent. We found 1.4-fold increased progranulin concentrations in patients with type 2 diabetes. Moreover, circulating progranulin levels correlate with BMI, total body fat mass, and visceral fat area, suggesting that progranulin reflects the chronic inflammation in obesity. We further found significant relationships between progranulin and A1C, total cholesterol, MCP-1, and high-sensitive CRP serum concentrations. The association between progranulin and CRP serum concentrations was independent of the significant relationships with BMI and A1C, whereas the correlation between progranulin and MCP-1 serum concentrations was not significant after adjustment for BMI. Our data therefore suggest a close coregulation between CRP and progranulin serum concentration and demonstrate that progranulin may be a novel biomarker of the chronic inflammatory response in central obesity and associated disturbances. This is further supported by the finding that changes in progranulin levels are best predicted by changes in CRP serum concentrations after a 4-week training program. Interestingly, CRP serum concentrations significantly improve after 4 weeks of training both in patients with IGT and type 2 diabetes (4), whereas significant reduction of progranulin was detectable only in the type 2 diabetic patients. We cannot exclude that the number of subjects in the NGT and IGT groups was not sufficient to detect significant changes of progranulin serum concentrations. The time course of progranulin serum concentration in response to acute and chronic exercise created a paradox. Progranulin serum concentrations increased immediately after exercise followed by a significant decrease 48 h after the exercise. Exercise is known to induce an increase in circulating cytokines (30). Exercise-induced increase in circulating progranulin could be a consequence of an immune response due to local damage in the working muscles. The exercise-induced progranulin time course is similar to the effect of acute exercise on plasma IL-6 (30), suggesting common exercise-related mechanisms for IL-6 and progranulin. Further studies are necessary to investigate whether increased progranulin serum concentrations are the cause or consequence of increased IL-6 in response to exercise.

Another key finding of our study was the identification of the significant relationship between progranulin serum concentrations and the amount of macrophages within adipose tissue, especially in the visceral depot. We and

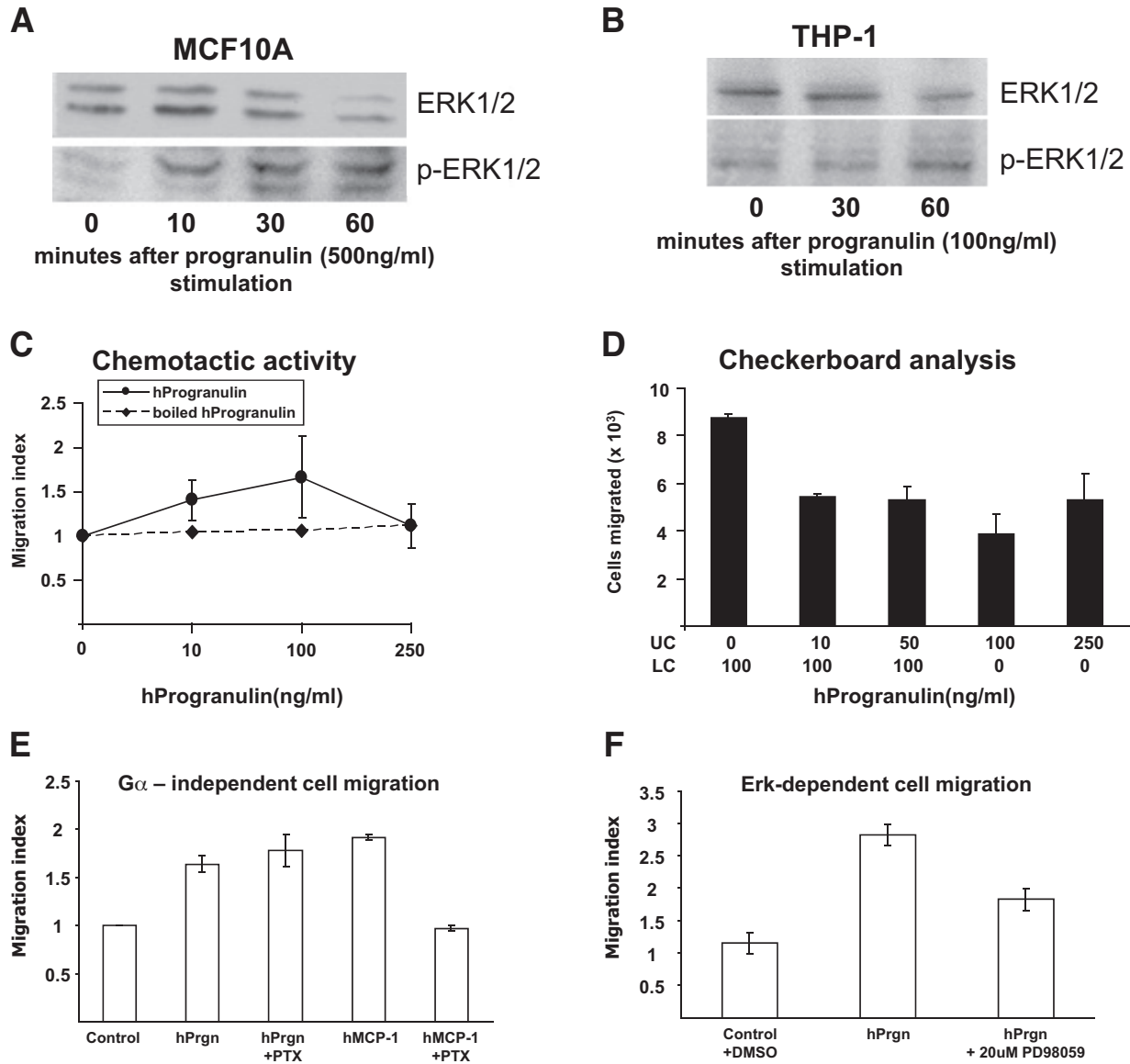


FIG. 4. Chemotactic activity exerted by recombinant human progranulin. *A:* Erk activation by recombinant human progranulin in MCF10A cells. To examine the signal of phospho-p44/42 MAPK and p44/42 MAPK, after serum starvation for 24 h, MCF10A cells were stimulated with 500 ng/ml progranulin. Cell lysates were prepared at different time points as indicated and subjected to Western blot. *B:* Similar results have been obtained after treatment of THP-1 with 100 ng/ml progranulin. *C:* Transwell migration assay using THP-1 cells was conducted as described in RESEARCH DESIGN AND METHODS. THP-1 cells were placed into upper chambers, setting out to cell migration to lower chambers harboring no progranulin, different concentrations of progranulin (10, 100, and 250 ng/ml), or boiled progranulin. After 16 h, migrated cells were counted. *D:* Chemotactic effect of human Progranulin-FLAG in THP-1 cells. Cell migration of THP-1 monocytic leukemia cells was evaluated in disposable 24-well transwell polystyrene membrane with 8- μ m pore size. hProgranulin-FLAG (100 ng/ml) was diluted in RPMI 1640 supplemented with 0.5% FBS and was then added to the low chamber. 0, 10, 50, 100, and 250 ng/ml hProgranulin were added into upper chamber together with THP-1 cells (2×10^5 cells per well). After 16-h incubation at 37°C in 5% CO₂ humidified atmosphere, migration cells in the lower chamber were counted. Migrated cells in two separate fields per well from duplicate wells were enumerated on a hemacytometer by means of light microscopy. UC, upper chamber; LC, lower chamber. *E* and *F:* Transwell migration assay comparing human progranulin (hPrgn) and human MCP-1 (hMCP-1) using PTX-treated or PD98059-treated THP-1 cells.

others have previously shown that macrophage infiltration into adipose tissue accompanies obesity, with a potential preferential infiltration into visceral (omental) compared with subcutaneous fat (17,18). However, the mechanisms for macrophage attraction and the higher recruitment of macrophages in omental than in subcutaneous adipose tissue are not fully understood. In animal models of obesity, a relationship has been shown between adipose cell size and the amount of macrophages in the stroma vascular fraction of white adipose tissue (WAT) (31). Canello et al. (17) suggested that in addition to adipose cell hypertrophy, depot-specific vascularization and/or innervation might explain distinct WAT infiltration with

immune cells. So far MCP-1 has been suggested as a circulating factor attracting macrophages in adipose tissue (16,19). The close association between circulating progranulin and macrophage infiltration into adipose tissue suggests progranulin as a novel chemoattractant protein recruiting monocytes into adipose tissue. Immunohistochemistry and expression studies revealed that both adipocytes and cells of the SVF of adipose tissue contribute to progranulin expression in adipose tissue. Our data therefore suggest that progranulin contributes to the cross-talk between adipocytes and macrophages in adipose tissue. Increased expression of progranulin in visceral fat could therefore represent a dysfunction of

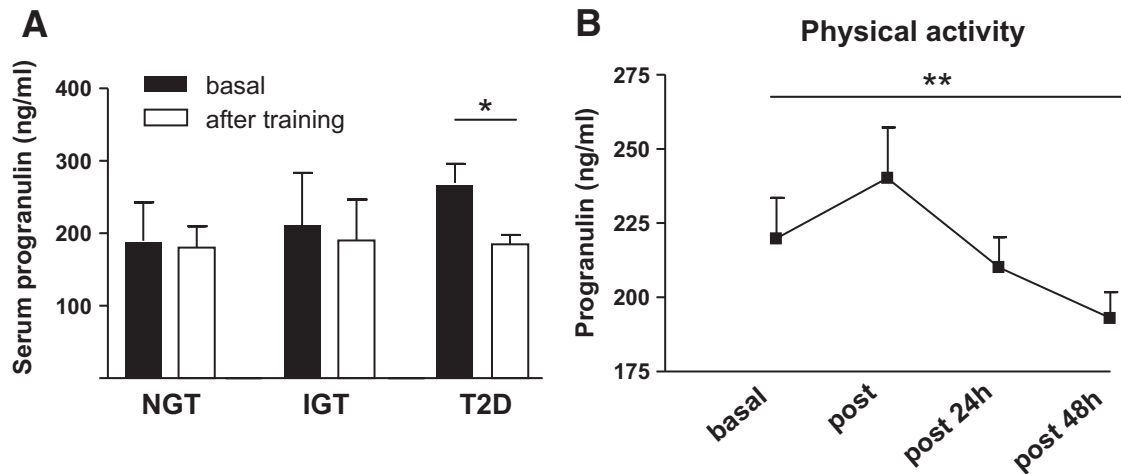


FIG. 5. Effect of 4 weeks of intensive exercise program on progranulin serum concentrations in NGT individuals and patients with IGT or type 2 diabetes (T2D). **A:** Circulating progranulin in groups of NGT ($n = 20$), IGT ($n = 20$), and type 2 diabetes ($n = 20$). **B:** Circulating progranulin before, immediately after, and 24 and 48 h after an acute exercise circle training to exhaustion for 60 min in 20 healthy volunteers. Data are means \pm SE. * $P < 0.05$ between baseline and after 4 weeks of intensive physical training. ** $P < 0.01$ between baseline and 48 hours after acute exercise.

adipocyte-macrophage interaction as a function of previously recognized increased macrophage numbers in omental depots (17,18). Although human adipose tissue macrophages have been shown to have an anti-inflammatory phenotype, they are capable of excessive proinflammatory mediator production (32). Macrophage-secreted factors, including TNF- α and IL-1 β , have been shown to induce adipocyte inflammatory response to a greater extent than the levels achieved by individual macrophage cytokines (33). Here, we identified progranulin as an additional secreted factor from both macrophages and adipocytes, which could induce or promote adipocyte inflammatory response and recruit monocytes into adipose tissue. We could demonstrate that in vitro chemotactic activity of progranulin using a human macrophage-like cell line, THP-1 cells, is indistinguishable from the so far best-established marker of adipose macrophage infiltration, MCP-1 (34). These data further support our hypothesis that progranulin represents a previously unrecognized chemoattractant molecule, which contributes to the higher number of adipose tissue macrophages in patients with central obesity. Similar to the previously reported higher MCP-1 release from visceral compared with subcutaneous fat depot (35), our observation of higher progranulin expression in visceral adipose tissue could contribute to predominant omental macrophage infiltration in human visceral obesity. However, unlike MCP-1, our data strongly suggest that progranulin-mediated chemotaxis depends on Erk, but not on G α , indicating that progranulin receptor(s), if any, may not be a G-protein-coupled receptor(s). Because of heterogeneity of tissue macrophages, including the obesity-induced omentum-macrophages, a potential contribution of progranulin to recruitment or activation of macrophages remains to be determined. This should also require identification of the cell surface markers specifically expressed in the omental macrophages. Nevertheless, progranulin could be an important homing factor for the omental macrophages.

In conclusion, our data suggest progranulin as a novel marker for chronic inflammation associated with visceral obesity and type 2 diabetes in humans. In addition, progranulin plays a role as a chemoattractant molecule,

which, similar to MCP-1, could contribute to increased macrophage infiltration in omental adipose tissue of patients with predominantly visceral obesity.

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