Introduction

Interleukin (IL)-18 was identified and classified as a member of the proinflammatory IL-1 family (1). IL-18, originally described as the interferon γ-inducing factor (IGIF), was cloned by us from the pancreas of animals with autoimmune type 1-like diabetes (2). Subsequently, a regulatory role of IL-18 in islet inflammation was demonstrated (3). Overproduction of IL-18 is known to induce severe inflammatory disorders (4). In general, IL-18 enhances the IL-12-driven T helper (Th) 1 immune response, but IL-18 is also able to stimulate Th2 immune responses in the absence of IL-12 (4–6). Several lines of evidence indicated recently that IL-18 is also involved in atherosclerosis. An elevated concentration of systemic IL-18 has been reported to be a strong predictor of cardiovascular death in a prospective study with patients who had documented coronary artery disease (7). IL-18 is also highly expressed in atherosclerotic plaques and seems to be a major factor in plaque destabilization (8). Importantly, levels of the proatherogenic cytokine IL-18 are known to be elevated in obesity (9, 10), which represents a key risk factor for the development of cardiovascular disease (11, 12). Obesity is accompanied by low-grade systemic inflammation, and it is probable that some of the circulating inflammatory mediators are released from obese fat tissue, from adipocytes and/or from macrophages within obese fat tissue (13–15). The relevance of obesity and body fat mass for systemic immune-mediator concentrations is supported by observations that weight loss attributable to alterations in diet and/or physical activity as well as liposuction decreased systemic and local cytokine concentrations in adipose tissue (16–18). Weight loss also caused a decrease of systemic IL-18 concentrations (9, 10). This finding led us to investigate in the current study whether human adipocytes produce and secrete IL-18. Because of its central role in regulating innate immunity (4), IL-18 could be a key mediator of subclinical inflammation associated with abdominal obesity and in particular represent a link between obesity and the increased risk of developing cardiovascular disease.

Subjects and methods

Subjects

Adipose tissue samples were obtained from mammary adipose tissue of lean to slightly overweight adult females (age < 40 years, body mass index (BMI)
<27 kg/m²) undergoing elective mammary reduction and from obese men and women undergoing laparoscopic surgery (gastric banding; age range 33–65 years. BMI ³ 30 kg/m²). All patients were otherwise healthy; in particular they were free of metabolic or endocrine diseases as assessed by routine clinical examination and laboratory tests. The procedure for obtaining human adipose tissue has been approved by the Ethical Committee of the Heinrich-Heine-University Düsseldorf.

**Cell culture**

Stromal cells from human adipose tissue were prepared as described previously (19). Briefly, after removing all fibrous material, gland tissue and visible blood vessels, adipose tissue samples were cut into pieces of 10–20 mg and digested in 10 mM PBS, 200 U/ml crude collagenase (Biochrom, Berlin, Germany) and 20 mg/ml BSA (Sigma, Munich, Germany), pH 7.4, for 60 min at 37°C in a shaking water bath. After centrifugation at 200 g for collection of disaggregated cells, supernatant with the adipocytes was aspirated and discarded or used for other experiments. Sedimented cells were purged of contaminating red blood cells, filtered through a 150-µm nylon mesh (VWR International, Darmstadt, Germany) and washed twice. The sedimented cells were resuspended in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (DMEM/F-12 medium; Life Technologies, Duesseldorf, Germany) and washed twice. The sedimented cells were suspended in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (DMEM/F-12 medium; v/v, 1:1) supplemented with 10% fetal calf serum (Bio-Eagle’s/Ham’s F-12 medium; Biowest, Nagoya, Japan) and washed twice. The sedimented cells were resuspended in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (DMEM/F-12 medium; v/v, 1:1) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and washed twice. The sedimented cells were resuspended in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (DMEM/F-12 medium; v/v, 1:1) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and washed twice. The sedimented cells were resuspended in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (DMEM/F-12 medium; v/v, 1:1) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and washed twice.

**Gene expression analysis by real-time reverse transcriptase PCR**

Total RNA of the cultured adipocytes (days 4–16 of culture) was isolated according to the PAXgene Blood RNA protocol (Qiagen, Hilden, Germany) and transcribed with Omniscript reverse transcriptase according to the manufacturer’s instructions (Qiagen). Gene-expression analysis was performed using the ABI 7700 sequence detection system (PE Applied Biosystems). Amplification products were measured based on SYBR Green fluorescence (Qiagen). The primers used (MWG-Biotech, Ebersberg, Germany) were 5′-GCC AGG AGA ATC ACT TGC ACT-3′ (forward) and 5′-AGA GCG CAA TGG TGC AAT C-3′ (reverse), amplifying a 67 bp product of human IL-18 (GenBank accession number NM_001562). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalize for the relative abundance of IL-18 transcripts. The primer sequences for GAPDH were 5′-CTT CTG ACT TCA ACA GGC ACA C-3′ (forward) and 5′-AGC TGG ACA AAG TGG TCG TTG AG-3′ (reverse), amplifying a 82 bp product (GenBank accession number M33197). Amplification was performed over 40 cycles (initial activation step, 15 min at 95°C; followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 45 s at 72°C). To get optimal results (cycle threshold number, Ct, between 20 and 30) we used 2.5 and 100 ng cDNA in the GAPDH and IL-18 assays, respectively.

**IL-18 determination**

IL-18 protein was detected by sandwich ELISA (MBL, Nagoya, Japan) according to the manufacturer’s instructions, but with incubation of standards and samples for 15-18 h to increase sensitivity.

**Immunocytochemistry**

Cells cultured on glass slides were washed with PBS, fixed with 80% methanol/20% acetone (v/v) for 1 h at −20°C and washed with Tris-buffered saline (8% NaCl and 2.4% Tris base, pH 7.6). IL-18 was detected with a monoclonal mouse anti-human IL-18 antibody (DO44-3; MBL) and the horseradish peroxidase-diaminobenzidine (HRP-DAB) system (R&D Systems, Minneapolis, MN, USA).

**Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity**

GPDH activity was assessed as a marker of adipocyte differentiation according to an established method (20). Cells were washed twice in PBS and harvested in a Tris buffer (0.05 M Tris/HCl, 1 mM EDTA and 1 mM mercaptoethanol, pH 7.4). After complete cell

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lysis by sonification and subsequent centrifugation. GPDH activity was assessed spectrophotometrically after addition of NADH and dihydroxyacetone phosphate (DHAP; Sigma). For protein determination an established method according to Lowry (21), including protein precipitation with trichloroacetic acid, was used. Specific activity was calculated and expressed by mU/mg protein.

Statistical analysis
Results are expressed as means±S.D. from three independent experiments in triplicates. For comparison ANOVA or t-test were used as indicated. P < 0.05 was considered to be statistically significant.

Results
IL-18 protein production in cultured preadipocytes and freshly isolated human adipocytes
In the first set of experiments, the IL-18 secretion of cultured or freshly isolated human adipocytes was investigated. To characterize the maturation stage of preadipocytes, GPDH activity was determined. There was a significant increase in GPDH activity over the culture period of 12 days, indicating differentiation from pre- to mature adipocytes from mammary adipose tissue (Fig. 1A). Mammary preadipocytes cultured in non-differentiating conditions (i.e. without troglitazone and IBMX) secreted IL-18 protein spontaneously without significant change during the culture period of 16 days (Fig. 1B). In the presence of troglitazone and IBMX, i.e. under culture conditions favoring adipocyte maturation, IL-18 secretion increased significantly with peak concentrations of 8.8±4.1 pg/ml at day 12 (Fig. 1C). Freshly isolated adipocytes from subcutaneous and omental depots from both male and female donors also released IL-18 which was found in culture supernatants at levels of 3.2±2.9 and 8.6±4.7 pg/ml, respectively (Fig. 1D). Both subcutaneous and omental adipocytes were available from four different donors. For these donors, IL-18 accumulation did not differ significantly between the depots. IL-18 concentrations in cultures of subcutaneous adipocytes from obese donors (BMI ≥ 30 kg/m²) reached 5.5±4.2 pg/ml and were higher than IL-18 concentrations in adipocyte cultures from non-obese donors with a BMI of < 30 kg/m² (1.8±0.9 pg/ml). However, the difference was not statistically significant (P = 0.09; Fig. 1E).

IL-18 mRNA expression in adipocytes
Expression of IL-18 was confirmed by PCR. mRNA was isolated from mammary adipocyte cultures during differentiation (days 4–16). IL-18 mRNA expression could clearly be demonstrated in preadipocytes during maturation (C_T 22.82±0.98; mean±S.D.). GAPDH was amplified as an mRNA quality control (C_T 21.89±0.14; n = 4 in both cases).

Immunocytochemistry
Immunocytochemical experiments revealed cytoplasmatic staining for IL-18 in both preadipocytes and freshly isolated mature adipocytes (Fig. 2). The strongest staining was seen in cells with lipid droplets, which does not support the possibility that contaminating cells of the stromal fraction are responsible for IL-18 production.

Discussion
Our study demonstrates that human adipocytes release IL-18 spontaneously. Cultures of human preadipocytes secreted small but detectable amounts of IL-18 into the medium. The amounts accumulating in the supernatant during 48 h were increased during differentiation from preadipocytes into mature fat cells, suggesting that the ability to release IL-18 is a property already expressed in precursor cells and augmented further throughout differentiation. During the differentiation period between 45 and 55% of the initially lipid-free preadipose cells acquired the adipocyte phenotype under the adipogenic culture conditions used. It is also interesting to note that freshly isolated mature omental fat cells were found to spontaneously release IL-18 to a similar extent as in vitro-differentiated fat cells at day 12. When RNA was extracted from in vitro-differentiated adipocytes, significant amounts of IL-18 mRNA could be measured by quantitative real-time PCR, which confirmed the data from the ELISA. Using immunocytochemical methods it was possible to stain IL-18 within lipid-containing fat cells. The cytokine was found throughout the cytoplasm of adipocytes, and also around lipid droplets. These findings link the IL-18 production observed to cells of the adipocyte phenotype. Another novel and potentially important finding is that we observed a trend towards upregulation of IL-18 release in adipocyte cultures from obese compared with lean or overweight donors. Our data may help to explain why systemic IL-18 concentrations seem to correlate directly to fat cell mass. Two recent studies reported a decrease of IL-18 plasma concentrations in obese individuals upon weight loss (9, 10). There was a significant positive correlation between weight loss or decreased waist-to-hip ratio and low systemic IL-18 concentrations suggesting that adipose tissue might be a source of circulating IL-18. IL-18 possesses a high inflammatory potency. Apart from its activity to stimulate interferon-γ release from macrophages IL-18 exhibits marked chemoattractive properties, especially to CD4⁺ T cells (22). Upregulation of IL-18 release in obesity as suggested in our study...
might thus contribute to increased leukocyte infiltration into adipose tissue and to the chronic proinflammatory state in obesity. Although either Th1 or Th2 responses can be driven by IL-18, sustained IL-18 receptor expression on Th1 cells suggests a preferential activation of the latter in chronic lesions (23). IL-18 is therefore a candidate which could direct the reaction of the immune system towards a Th1-associated proinflammatory state.

It will be important to study whether adipocytes express IL-18 receptors and therefore would respond to its own product. IL-18 may also promote the detrimental effects of obesity by inducing insulin resistance in hepatocytes or muscle cells, as has been reported for other proinflammatory cytokines (24–26). A close association between systemic concentrations of IL-18 and atherogenesis is supported by reports of circulating IL-18 as a strong predictor of cardiovascular death in patients with stable and unstable angina (7). Overexpression of IL-18 was found to occur in human atheroma in situ, and due to the positive correlation between IL-18 expression and plaque instability a pathogenetic role of the cytokine atherosclerosis has been postulated (8). In animal studies based on apolipoprotein E–/– mice, administration of exogenous IL-18 led to increased lesion size and augmented numbers of lesion-associated T cells and antigen-presenting cells (27), whereas expression of the inhibitory IL-18 binding protein or disruption of the IL-18 gene reduced progression of plaque development and destabilization (28, 29). There is evidence that the proatherogenic activity of IL-18 might be mediated by action on atheroma-associated endothelial cells, macrophages and smooth muscle cells, which all express the IL-18 receptor and are able to respond to IL-18 stimulation (8, 30). In particular, incubation of human cardiac microvascular endothelial cells with IL-18 could be shown to activate proapoptotic pathways and induce endothelial cell death (31). The proatherogenic potential of IL-18 has therefore been documented by a wide range of observations, and the characterization of its cellular sources might allow for more targeted interventions in cardiovascular therapy. In summary, the data presented here demonstrate that human preadipocytes and mature adipocytes secrete IL-18. There was a trend towards higher IL-18 release from adipocytes from obese donors compared to non-obese donors. The release of IL-18 from adipose tissue may be one of the pathogenetic factors linking obesity with cardiovascular disease and other related disorders, such as type 2 diabetes.
Figure 2 Expression of IL-18 protein in adipocytes from mammary adipose tissue by immunocytochemistry. (A, B) Preadipocytes without and with secondary antibody, respectively (original magnifications, ×20 and ×40 respectively). (C, D) Differentiated fat cells without and with secondary antibody, respectively (original magnifications, ×40). The strongest staining was seen in cells with lipid droplets.

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References

2 Rothe H, Jenkins NA, Copeland NG & Kolb H. Active stage of autoimmune diabetes is associated with the expression of a novel cytokine, IGIF, which is located near Idd2. Journal of Clinical Investigation 1997 99 469–474.
4 Liew FY. The role of innate cytokines in inflammatory response. Immunology Letters 2003 85 131–134.
20 Pairault J & Green H. A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. PNAS 1979 6 5138–5142.

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