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The adiponectin paralog CORS-26 has anti-inflammatory properties and is produced by human monocytic cells

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Abstract The adiponectin paralog CORS-26 (collagenous repeat-containing sequence of 26 kDa protein) is a member of the C1q/TNF-α molecular superfamily. CORS-26 is a secreted protein and baculovirus-produced CORS-26 released in the supernatant of insect cells forms stable trimers. Adiponectin exerts anti-inflammatory effects in LPS-treated monocytic cells and CORS-26 also reduces IL-6 and TNF-α secretion but does not increase IL-10. Suppression of NFκB signalling may explain the anti-inflammatory actions of CORS-26. Furthermore CORS-26 protein was detected in human monocytic and dendritic cells. The present data demonstrate for the first time that CORS-26 forms trimers, exerts anti-inflammatory properties and that it is expressed in monocytic cells. Therefore CORS-26 may provide a new target for pharmacological drugs in inflammatory diseases like the metabolic syndrome.

Keywords: Adiponectin; Macrophage; Inflammation

1. Introduction

Human and murine CORS-26 (collagenous repeat-containing sequence of 26 kDa protein) are 246 amino acid proteins with a secretory signal peptide, a collagenous region and a globular C-terminal domain [1]. CORS-26 shows striking homologies to the adipocyte-specific secretory protein adiponectin, both belonging to the newly discovered C1q/TNF family [1]. Adiponectin is a highly abundant circulating adipokine secreted by differentiated adipocytes and is decreased in obese and diabetic humans and animals [2]. Adiponectin has anti-inflammatory and antidiabetic effects which was demonstrated in numerous in vitro studies and in animal models [3]. Adiponectin forms trimers, hexamers and higher molecular weight (HMW) complexes, and these isoforms have been detected in sera and conditioned media of differentiated 3T3-L1 adipocytes. CORS-26 mRNA expression was also found induced in hormonally differentiated 3T3-L1 cells and the mRNA was detected in human synovial adipocytes of the knee by in situ hybridization [4]. Whereas adiponectin is exclusively expressed by adipocytes, CORS-26 mRNA was also detected in cartilage, kidney [1], colon, small intestine, placenta and fibroblasts [5]. In addition, a strong CORS-26 mRNA expression was found in osteosarcoma, chondroblastoma and giant cell tumor. Together with its chromosomal location on a candidate locus for osteosarcoma, these data indicate a role for CORS-26 in the context of mesenchymal tissue development and in the pathogenesis of bone or skeletal disease [5].

Transfection analysis using the green monkey kidney cell line COS-7 confirmed that CORS-26 is a secretory protein. Recombinant CORS-26 was detected as a 26 kDa protein in the cell lysate and the supernatant of these cells. Overexpression of CORS-26 in C3H10T1/2, a murine mesenchymal stem cell line devoid of CORS-26 mRNA, significantly enhanced cell growth and furthermore increased saturation densities of the cultivated cells [1].

The high homology of CORS-26 and adiponectin led us to investigate whether CORS-26 also forms HMW forms. In addition the influence of recombinant CORS-26 on the secretion of IL-6, TNF-α and IL-10 in LPS-stimulated monocytic cells was analyzed.

2. Materials and methods

2.1. Material

RPMI medium was obtained from Gibco BRL (Karlsruhe, Germany) and DMEM medium from PAA Laboratories (Pasching, Austria). LPS, *Escherchia coli* serotype 055:B5, and other laboratory reagents and chemicals were purchased from Sigma Chemical (Deisenhofen, Germany). Oligonucleotides were synthesized by Metabion (Planegg-Martinsried, Germany). DuoSet ELISA Development System for human IL-6, IL-10 and TNF-α ELISA were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). Recombinant M-CSF was from R&D Systems. The Cytotoxicity Detection Kit (Roche, Penzberg, Germany) was used to determine lactate dehydrogenase (LDH) activity in the supernatants. Antibodies to CORS-26 were raised in rabbits using a peptide located in the collagenous domain of the protein (*CYS*YEMKGKSDTSSNH, CORS-26 specific amino acids are underlined; Pineda, Berlin, Germany). Antibody to NFκB p65 was obtained from New England Biolabs (Frankfurt am Main, Germany). 6× HN-tag antibody was from BD Biosciences (Palo Alto, CA, USA). Human tissue immunoblot was from Biomol International (Exeter, United Kingdom). Ready Prep Protein Extraction Kit (Bio-Rad, Hercules, CA, USA) was used to isolate nuclear protein.

2.2. Expression and purification of adiponectin and CORS-26 using insect cells

Full-length adiponectin and CORS-26 cDNA (NM_004797.1 and NM_030945) were amplified with the primers 5′-ggg**ggatcc**t-tatgctgttgctgggagctgttcta-3′ and 5′- ggg**tctaga**ccgttggtgtcatggtagagaaga aagcc-3 or 5′-ggg**ggatcc**ttatgctttggaggcagctcatctattgg-3′ and 5′-ggg**tct-**

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agacccttagtttcaaaga gcaggaatcc-3′ from human mRNA isolated from adipose tissue (Biocat, Heidelberg, Germany) and were cloned in frame with the 6× HN tag in the pDNR Dual Donor vector (BD Biosciences) using the *Bam*HI and *Xba*I sites (bold in the primer sequence). After sequencing, the cDNA was transferred to the BacPAK9 vector using the Cre/lox procedure (BD Biosciences). Recombinant baculoviruses were generated by the BacPAK™ Baculovirus Expression System (BD Biosciences, Palo Alto, CA, USA). Insect H5 cells (Invitrogen, Karlsruhe, Germany) were infected with the recombinant viruses and supernatant was collected after 3 d for purification of adiponectin or CORS-26 with the BD Talon™ Purification kit (BD Biosciences). Integrity and purity of the purified proteins was analyzed by immunoblot and silver staining of SDS–PAGE.

2.3. Cultivation of cells

THP-1, COS-7, CHO, Caco-2 and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). THP-1 were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Biochrom, Southborough, MA, USA) and incubated in 10% CO_2 in air at 37°C. To induce phagocytic differentiation, THP-1 cells were cultured in the presence of 160 nM PMA for 24 h. COS-7, CHO, Caco-2 and HeLa cells were cultivated in DMEM medium with 10% fetal calf serum. Monocytic and dendritic cells were isolated and cultivated as described previously [6,7]. Monocytes were cultivated for 24 h in RPMI medium with 10% fetal calf serum. Subsequently medium was replaced and recombinant CORS-26 was added as indicated. After 1 h preincubation LPS at a concentration of 1 ng/ml was added. The study protocol was approved by the local ethics committee and was carried out in accordance with the Helsinki guidelines.

2.4. Monitoring of gene expression by real-time RT-PCR

Total cellular RNA was isolated, reverse transcribed and used in real-time RT-PCR as described recently [8] using primers specific for CORS-26 and β-actin. The primers for β-actin were: β-actin uni 5′-ccagggtgtgatggtgggaatg-3′, β-actin rev 5′-cgcacgatttccctctcagctc-3′. The primers for CORS-26 were: CORS-26_436-uni 5′-ttgcattcatggcttctctg-3′, CORS-26_649-rev 5′-actgtgttgccattgtgcat-3′.

2.5. SDS–PAGE and immunoblotting

The cells were harvested, washed in PBS, and solubilized in RIPA buffer. Proteins were separated by SDS–polyacrylamide gel electrophoresis and were transferred to PVDF membranes. Incubations with antibodies were performed in 5% non-fat dried milk in TBS, 0.1% Tween. Detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham Pharmacia, Deisenhofen, Germany).

2.6. ELISA

300 000 THP-1 cells or primary monocytes were incubated for the times indicated. The supernatants were used for IL-6, TNF-α and IL-10 protein determinations. The ELISAs were performed as recommended by the distributor (R&D Systems, Wiesbaden-Nordenstadt, Germany).

2.7. Statistics

Data are represented as Box Plots indicating the mean, the upper and lower quartile, the highest and the lowest value in the data set (SigmaPlot 8.0). Variances of the data sets were compared by *F* test (MS Exel). Statistical differences were analyzed by paired, two-tailed Student's *t* test and a value of $P < 0.05$ was regarded as statistically significant (MS Exel).

3. Results

3.1. Baculovirus-mediated recombinant expression of CORS-26

Recombinant CORS-26 was expressed in insect cells as described in material and methods. Intracellular CORS-26 separated by SDS–PAGE using reducing buffers and heat-denatured protein samples was detected as a 26 kDa protein by immunoblot with an antibody to the 6× HN-tag (Fig. 1A). Intracellular CORS-26 was similar in size when compared to full-length adiponectin expressed in H5 cells. Lysates from H5 cells either expressing adiponectin, CORS-26 or the collagenous region of adiponectin were separated by SDS–PAGE as described above and used in immunoblot with an antiserum raised against a CORS-26 specific peptide in rabbits. A signal was only detected in CORS-26 expressing cell lysates indicating that the antiserum is specific for CORS-26 (Fig. 1B). CORS-26 was purified from the supernatants of infected H5 cells. Purified protein was analyzed by non-reducing and non-heat-denaturing conditions by SDS–PAGE and had a molecular weight of about 90 kDa (Fig. 1C) most likely resembling trimeric CORS-26. Simple SDS–PAGE under non-reducing and non-heat-denaturing conditions was reported to be suitable to separate adiponectin isoforms [9] and recombinant adiponectin purified from the supernatant of H5 cells also forms trimers (Fig. 1C). Whereas the antibody to the 6× HN-tag detects trimeric CORS-26, the CORS-26 specific antiserum was not able to bind to trimeric CORS-26 most likely because the peptide used for immunization is not accessible when the trimer is being formed (not shown). Reducing buffers and incubation of the protein at 95 °C for 5 min did not convert the trimeric CORS-26 to the monomeric form (not shown).

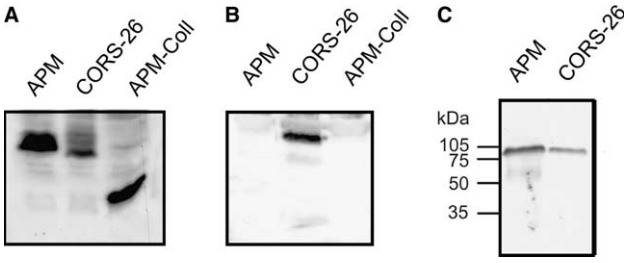

Fig. 1. Analysis of baculovirus expressed intracellular and secreted CORS-26 and adiponectin (A) Baculovirus expressed CORS-26, adiponectin (APM) and the collagenous region of adiponectin (APM-Coll) were incubated at 95 °C for 5 min and separated by SDS–PAGE under reducing conditions. Subsequently an immunoblot using a 6× HN tag antibody or (B) a CORS-26 specific antiserum raised in a rabbit was performed. (C) Non-reducing SDS–PAGE of adiponectin and CORS-26 purified from the supernatant of transfected H5 insect cells without incubation at 95 °C. Protein was transferred to PVDF membrane and immunoblot was done with the 6× HN tag antibody.

3.2. Antiinflammatory effects of recombinant CORS-26 in THP-1 cells

Antiinflammatory effects of recombinant adiponectin (1 and 10 μg/ml protein were used), have been described in primary monocytes and macrophages [10,11]. THP-1 cells differentiated with 160 nm PMA for 24 h were used to study the influence of recombinant CORS-26 on LPS-mediated cytokine secretion. Differentiated THP-1 cells were incubated with LPS and either 250, 500 or 1000 ng/ml recombinant CORS-26 for 18 h. IL-6 in controls was 965 ± 29 pg/ml. 250 ng/ml CORS-26 reduced IL-6 to 788 ± 35 pg/ml, 500 ng/ml CORS-26 to 838 ± 100 pg/ml and 1000 ng/ml to 788 ± 36 pg/ml (Fig. 2A). These experiments indicate that recombinant CORS-26 significantly reduces IL-6 release in LPS-treated THP-1 cells. There was no difference in the effect of 250, 500 or 1000 ng/ml CORS-26. Similar results were

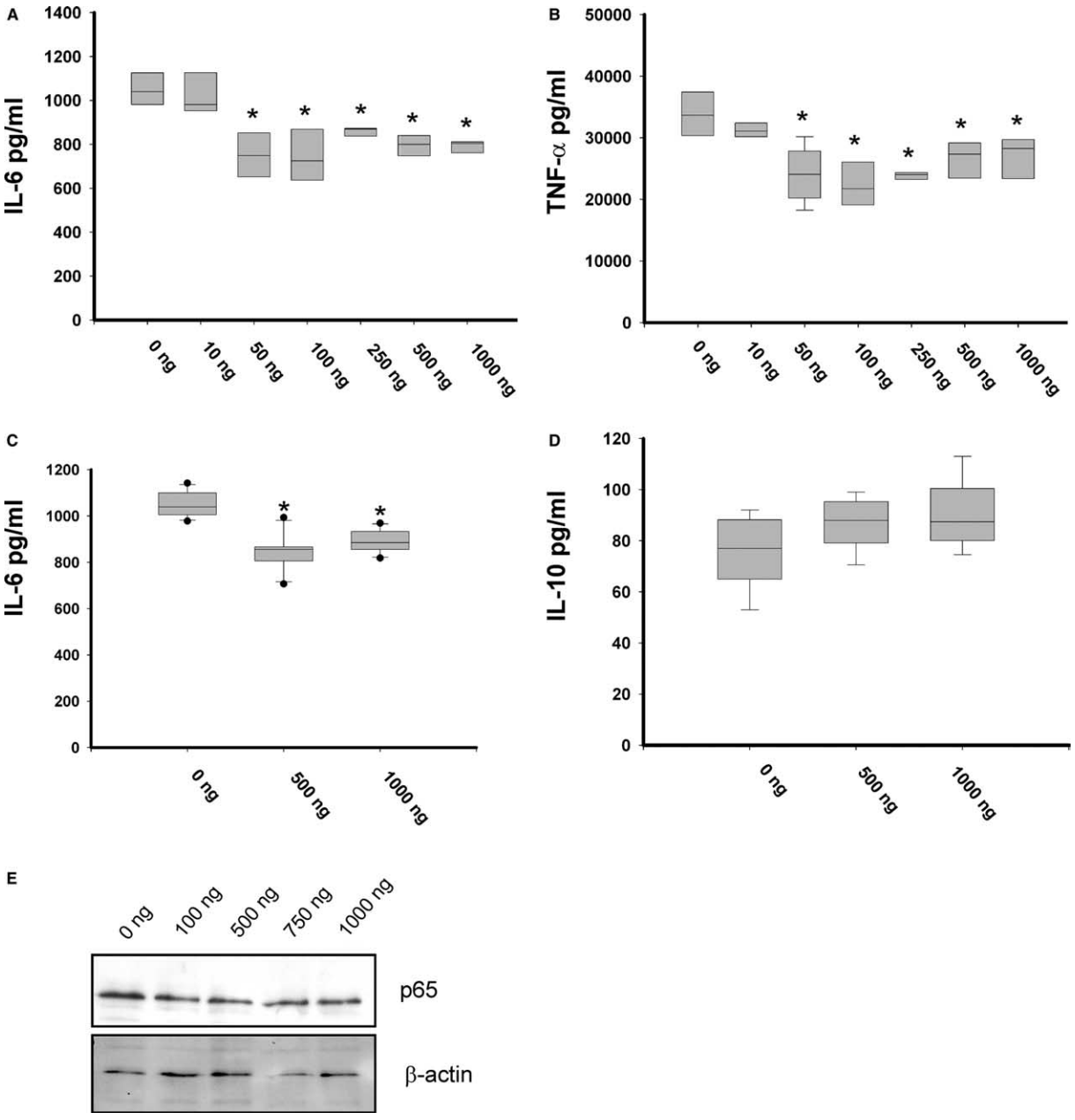

Fig. 2. Effect of recombinant CORS-26 on LPS-induced secretion of IL-6 and TNF-α in THP-1 cells and LPS-induced IL-6 and IL-10 release in primary monocytes. 300 000 PMA-differentiated THP-1 cells were incubated with 1 μg/ml LPS alone or in combination with CORS-26 (10, 50, 100, 250, 500 or 1000 ng/ml) for 18 h. IL-6 (A) and TNF-α (B) were determined in the supernatants. At least three independent experiments have been performed. (C) Primary monocytes were incubated with LPS (1 ng/ml) and either 500 or 1000 ng/ml recombinant CORS-26 for 18 h and IL-6 was determined in the supernatants. Data are given from three cultures of three different donors. (D) Primary monocytes were incubated with LPS (1 ng/ml) and either 500 or 1000 ng/ml recombinant CORS-26 for 22 h and IL-10 was determined in the supernatants. Data are given from three cultures of three different donors. *Significance was calculated by Student's *t* test. (E) PMA-differentiated THP-1 cells were incubated with different amounts of CORS-26 for 1 h. 1 μg/ml LPS was added and cells were cultivated for additional 20 min. NFκB p65 and β-actin were determined in nuclear extracts isolated from these cells. On representative immunoblot of two independent experiments is shown.

obtained when TNF-α was determined in these supernatants. Control cells released 33 896 ± 4460 pg/ml. 250 ng/ml CORS-26 reduced TNF-α to 23 805 ± 747 pg/ml, 500 ng/ml CORS-26 to 26 300 ± 3907 pg/ml and 1000 ng/ml to 26 552 ± 4801 pg/ml (Fig. 2B). Therefore the experiment was repeated using lower amounts of recombinant CORS-26. IL-6 in controls was 1061 ± 26 pg/ml, addition of 10 ng/ml recombinant CORS-26 failed to reduce IL-6 (1107 ± 41 pg/ml), 50 ng/ml CORS-26 reduced IL-6 to 864 ± 75 pg/ml and 100 ng/ml to 809 ± 42 pg/ml (Fig. 2A). TNF-α in control cells was 48 935 ± 13 610 pg/ml. 10 ng/ml CORS-26 reduced TNF-α to 35 436 ± 9342 pg/ml, 50 ng/ml CORS-26 to 24 087 ± 4754 pg/ml and 100 ng/ml to 22 490 ± 4664 pg/ml (Fig. 2B). Whereas 10 ng/ml CORS-26 did not significantly suppress cytokine secretion, 50 ng/ml significantly diminished IL-6 and TNF-α release. When

recombinant adiponectin was used in these experiments 250 ng/ml protein were needed to significantly suppress IL-6 and TNF-α release (not shown).

Reduced cytokine secretion may be explained by a cytotoxic effect of recombinant CORS-26. Cell number and LDH activity was determined in THP-1 cells cultivated for 24 h with 1000 ng/ml CORS-26. Cell number and LDH was similar in controls and CORS-26 treated cells and therefore a cytotoxic effect of CORS-26 was excluded.

3.3. Effect of recombinant CORS-26 on IL-6 and IL-10 in primary monocytes

Primary monocytes were isolated from three different donors and incubated with LPS (1 ng/ml) and either 500 or 1000 ng/ml recombinant CORS-26 for 18 or 22 h. IL-6 was determined in the 18 h supernatants and was 1052 ± 59 pg/ml in controls, 847 ± 93 pg/ml in monocytes treated with 500 ng/ml CORS-26 ($P = 0.006$) and 845 ± 132 ng/ml in monocytes incubated with 1000 ng/ml recombinant protein ($P = 0.001$) (Fig. 2C). IL-10 was measured in the supernatants of primary monocytes treated with LPS and CORS-26 for 22 h. Although IL-10 levels are slightly higher in monocytes incubated with LPS and CORS-26, the increase is not significant ($P = 0.08$ for 1000 ng/ml CORS-26) (Fig. 2D). A similar result was obtained when IL-10 was determined in the supernatants of THP-1 cells (not shown).

3.4. CORS-26 reduces nuclear translocation of NFκB p65

PMA-differentiated THP-1 cells were incubated with 100, 500, 750 or 1000 ng/ml recombinant CORS-26 or without CORS-26 for 1 h. 1 μg/ml LPS was added and cells were cultivated for additional 20 min. NFκB p65 was determined in nuclear extracts isolated from these cells and was found similarly reduced in THP-1 cells treated with different amounts of recombinant CORS-26 when compared to cells treated only with LPS (Fig. 2E).

3.5. mRNA and protein expression of CORS-26 in different cell lines and tissues

Expression of CORS-26 protein was investigated in several cell lines and human tissues using immunoblot. Hybridization of a human multiple tissue blot revealed abundance of CORS-26 in all tissues investigated with very high levels in lung and spleen (Fig. 3A). CORS-26 was found expressed in the epitheloid carcinoma cell line HeLa, the monocytic cell line THP-1 and the green monkey kidney cell line COS-7. CORS-26 was not detected in the Chinese hamster ovary cell line CHO and the colon carcinoma cell line Caco-2 (Fig. 3B). Therefore THP-1 cells do not only respond to recombinant CORS-26 but also produce CORS-26 protein.

Monocytes can be differentiated to macrophages or dendritic cells in vitro [6]. PMA-induced phagocytic differentiation of THP-1 did not alter CORS-26 mRNA or protein (not shown). Furthermore CORS-26 mRNA is similar in THP-1 cells, primary monocytes and immature dendritic cells (iDC) but is nearly 3-fold downregulated in mature dendritic cells (DC)(Fig. 3C). CORS-26 protein is low abundant in primary monocytes and strongly induced in differentiated macrophages. iDC have similar levels of CORS-26 than macrophages whereas it is lower in DC (Fig. 3D).

3.6. Influence of LPS and recombinant adiponectin on intracellular CORS-26 in THP-1 cells

Because CORS-26 has anti-inflammatory properties, the influence of LPS on CORS-26 protein abundance in THP-1 cells was investigated. However, CORS-26 protein was not altered by LPS (1 μg/ml) incubation for 1, 2, 3 or 18 h (Fig. 3E). Furthermore we tested whether recombinant adiponectin induces CORS-26 in THP-1 cells and thereby may exert its anti-iflammatory properties. Incubation with 1 μg/ml or 10 μg/ml recombinant adiponectin for 24 h did not influence CORS-26 mRNA (not shown) nor protein (Fig. 3F).

4. Discussion

CORS-26 is produced in adipose tissue [4] and therefore belongs to the growing family of adipokines, proteins released by adipose tissue [12]. Adiponectin is exclusively secreted by adipocytes and controls metabolism by enhancing insulin-sensitivity [13]. CORS-26 and adiponectin protein have a highly homologous structure characteristic for the family of C1q/TNF-α-related proteins currently consisting of 25 members [14]. Biologically active adiponectin forms trimers [9] and CORS-26 purified from the supernatant of H5 insect cells is detected as a protein of about 90 kDa most likely also resembling trimeric CORS-26. Trimeric adiponectin and CORS-26 were only detected in the supernatants whereas intracellularly only monomeric protein was present. Therefore we conclude that the 6× HN-tag may not artificially induce trimerization of recombinant CORS-26. Although adiponectin from higher eukaryotic cells forms hexameric and multimeric forms, baculovirus produced recombinant adiponectin is solely detected as a trimeric protein (own unpublished observation). Whether CORS-26 also forms higher multimers has to be analyzed in future studies.

Adiponectin exerts anti-inflammatory effects by reducing the release of IL-6 and TNF-α and increasing IL-10 in LPS-treated monocytes [10]. CORS-26 also significantly inhibits LPS-induced IL-6 and TNF-α secretion in PMA-differentiated THP-1 and IL-6 release in primary monocytes cells whereas IL-10 was slightly but not significantly elevated by CORS-26. Adiponectin [10] and CORS-26 reduce NFκB p65 activity. Therefore besides their structural homology both adiponectin and CORS-26 reduce the release of proinflammatory cytokines.

Whereas adiponectin expression is restricted to adipocytes, CORS-26 mRNA was also detected in cartilage, kidney [1], colon, small intestine, placenta and fibroblasts [5]. CORS-26 protein was detected in several human tissues and was most abundant in the lung and the spleen. CORS-26 mRNA was not detected in the lung, spleen or leukocytes by hybridization of a multiple tissue blot [5] most likely due to the lower sensitivity of this method when compared to RT-PCR techniques where CORS-26 mRNA was easily amplified from total RNA isolated from monocytes.

CORS-26 protein was also found in the green monkey kidney cells COS-7. Furthermore protein is produced in the epitheloid carcinoma cell line HeLa and the monocytic cell line THP-1. Although the CORS-26 specific antiserum specifically detects intracellular CORS-26, the antiserum did not react with secreted protein. Therefore it was not possible to confirm that these cell lines release CORS-26 to the supernatant.

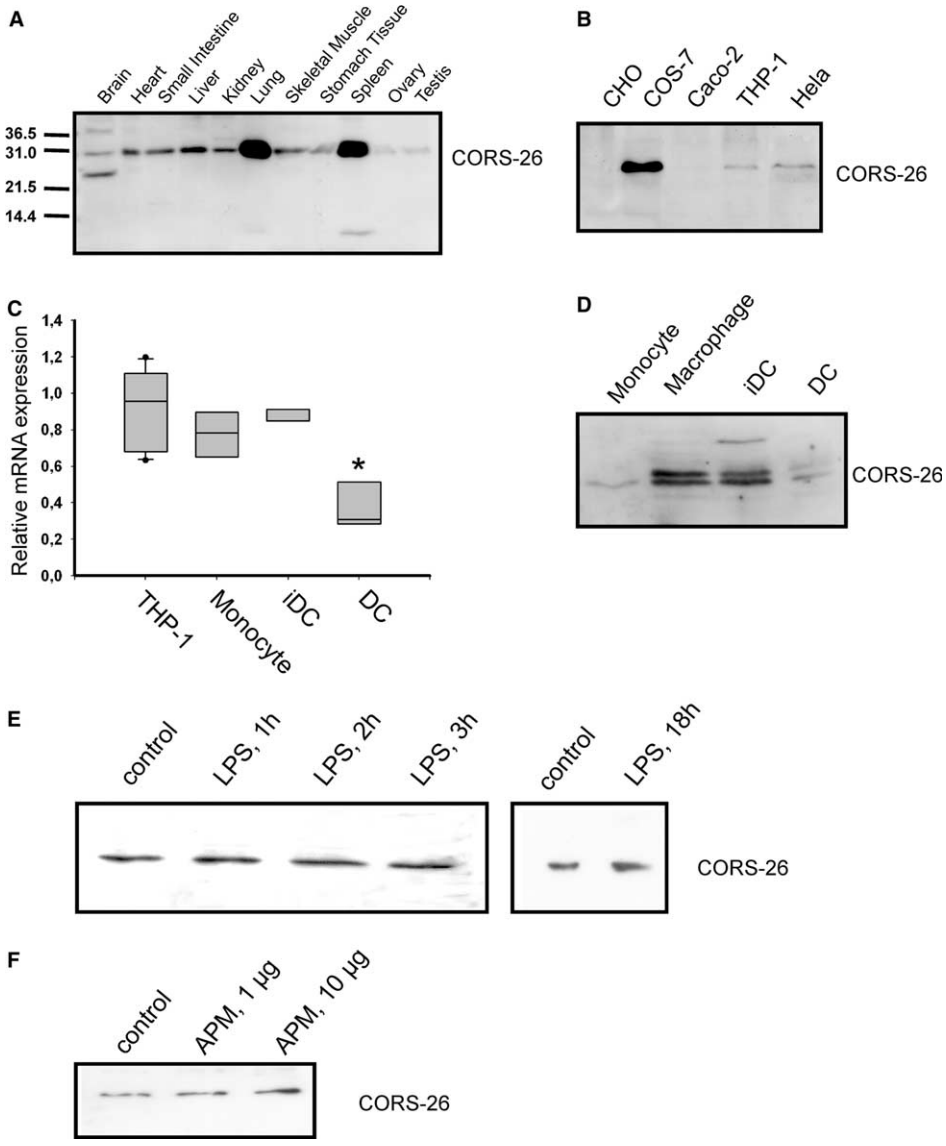

Fig. 3. Expression of CORS-26 in different cell lines and tissues and influence of LPS and recombinant adiponectin on CORS-26 in THP-1 cells. (A) The expression of CORS-26 was analyzed by immunoblot in different human tissues. (B) The expression of CORS-26 was analyzed by immunoblot in lysates from CHO, COS-7, Caco-2, THP-1 and HeLa cells. (C) CORS-26 mRNA was determined in THP-1 cells, primary monocytes, iDC and mature DC by real-time RT-PCR. (D) CORS-26 protein was analyzed in primary monocytes, macrophages, iDC and mature DC. (E) The expression of CORS-26 was analyzed by immunoblot in lysates of THP-1 cells treated with 1 µg/ml LPS for 1, 2, 3 and 18 h. (F) Expression of CORS-26 in THP-1 cells incubated with 1 or 10 µg/ml recombinant adiponectin for 24 h.

Because transfected COS-7 [1] and H5 insect cells secrete CORS-26 it is likely that these cell lines also release CORS-26. However, this has to be proven using a suitable antibody.

Taken together, our studies demonstrate that baculovirus-derived CORS-26 forms trimers and exerts anti-inflammatory effects. Therefore CORS-26 is a structural and functional adiponectin paralog. In contrast to adiponectin, CORS-26 is expressed in a variety of cells and tissues.

As adiponectin is regarded increasingly as a potential target for therapeutic approaches in various metabolic and inflammatory diseases such as diabetes mellitus type 2 [15], detailed knowledge of the adiponectin paralogs, like CORS-26, could provide new targets for the development of novel treatment strategies.

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