Adipocyte Macrophage Colony-stimulating Factor Is a Mediator of Adipose Tissue Growth

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Abstract

Adipose tissue growth results from de novo adipocyte recruitment (hyperplasia) and increased size of preexisting adipocytes. Adipocyte hyperplasia accounts for the severalfold increase in adipose tissue mass that occurs throughout life, yet the mechanism of adipocyte hyperplasia is unknown. We studied the potential of macrophage colony-stimulating factor (MCSF) to mediate adipocyte hyperplasia because of the profound effects MCSF exerts on pluripotent cell recruitment and differentiation in other tissues. We found that MCSF mRNA and protein were expressed by human adipocytes and that adipocyte MCSF expression was upregulated in rapidly growing adipose tissue that encircled acutely inflamed bowel and in adipose tissue from humans gaining weight (4–7 kg) with overfeeding. Localized overexpression of adipocyte MCSF was then induced in rabbit subcutaneous adipose tissue in vivo using adenoviral-mediated gene transfer. Successful overexpression of MCSF was associated with 16-fold increases in adipose tissue growth compared with a control adenovirus expressing β-galactosidase. This occurred in the absence of increased cell size and in the presence of increased nuclear staining for MIB-1, a marker of proliferation. We conclude that MCSF participates in adipocyte hyperplasia and the physiological regulation of adipose tissue growth. (*J. Clin. Invest.* 1998. 101:1557–1564.)
Key words: adipose tissue • macrophage colony-stimulating factor • obesity • gene transfer • adenovirus

Introduction

Adipose tissue is deposited in all mammals and grows via increases in adipocyte size (hypertrophy) and number (hyperplasia). Because adipocytes exhibit finite size, the severalfold increase in adipose tissue mass that occurs throughout life is primarily accounted for by hyperplasia. Adipocyte hyperplasia results from the recruitment of new adipocytes from pluripotent precursor cells (possibly fibroblast in origin), as adipocytes are unable to divide (1, 2). The mechanism of adipocyte hyperplasia is unknown. It is likely that adipocyte hyperplasia occurs in a localized fashion; adipocytes are not randomly distributed in mammals but are organized in identifiable depots. In addition, adipose tissue growth appears to occur within these depots through local recruitment of pluripotent precursors (3, 4) rather than through generation of new loci.

We elected to test the hypothesis that macrophage colony-stimulating factor (MCSF)[1] mediates adipocyte hyperplasia and adipose tissue growth for several reasons. First, MCSF exerts profound effects on recruitment and terminal differentiation of pluripotent cells in other systems, including other fibroblast-derived cells (5, 6); second, in cell culture systems, MCSF becomes downregulated once adipocyte terminal differentiation occurs (7); third, the paracrine nature of MCSF activity in other systems is compatible with the existing model of adipose tissue growth. Thus, the profound paracrine effects that MCSF exerts on recruitment and terminal differentiation of pluripotent cells in other tissues was reminiscent to us of how adipose tissue grows. Evidence was first gathered to ascertain whether adipocyte MCSF expression mirrored changes in adipocyte and adipose tissue growth. Adipocyte MCSF was then overexpressed in rabbit subcutaneous fat in vivo to determine whether MCSF might directly stimulate adipocyte hyperplasia and adipose tissue growth.

Methods

Identification of MCSF mRNA and protein in human adipocytes

To identify whether MCSF mRNA was expressed by human adipocytes, 10 g of fresh subcutaneous adipose tissue was obtained from a 72-kg woman undergoing surgery. Adipocytes were isolated (8) by incubating the tissue pieces for 20 min at 37°C in a solution of 0.05% collagenase, 4% nuclease-free BSA, and 0.5 mM glucose diluted in PBS. Collagenase digestion was followed by two steps of: centrifugation (200 $g \times 2$ min), floatation, and resuspension in PBS/glucose. Total RNA was isolated by homogenization (30 s), isovolume chloroform extraction, and application of the spun (10,000 $g \times 10$ min) supernatant to RNAeasy columns. Northern blot analysis was performed using 15 μg of total RNA, separated using denaturing agarose gel (1%) electrophoresis, and transferred to a positively charged nylon membrane. The membrane was hybridized with a psoralen-biotin–labeled antisense RNA probe for base pairs 845–1460 of MCSF mRNA (GenBank accession number M64592) (5 ng/ml) for 10 h at 65°C.

Total RNA was isolated in duplicate from human adipocytes (75-kg male) and from subcutaneous rabbit adipocytes (3.5-kg male New Zealand White rabbit) and treated with DNase (RNase-free; 50 U/μg) for 15 min at 21°C and then the DNase was inactivated by heating at 65°C for 15 min. Reverse-transcription (RT) was carried out on 200 ng of DNase-treated RNA using random hexamers in the presence and absence of reverse transcriptase. PCR was carried out using 10% of the RT products and using duplicate 2-μl vol of a 1:10,000 dilution of cDNAs obtained from a human adipocyte library (controls without

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1. *Abbreviations used in this paper:* LPL, lipoprotein lipase; MCSF, macrophage colony-stimulating factor; RT, reverse-transcription.

the library cDNAs were included) using primers specific for the human MCSF mRNA exon (forward primer, nucleotides 805–826, and reverse primer, nucleotides 1077–1097 of MCSF mRNA) for 35 cycles (denaturing 95°C, annealing 62°C, and elongation 72°C; terminal elongation 4 min). The products were separated using agarose gel (1.5%) electrophoresis, stained with ethidium bromide, and photographed using ultraviolet light.

Western blotting was performed on whole-cell isolates from human adipocytes. Whole cell isolates were prepared using three freeze-thaw cycles, sonication, and lipid separation by floatation. Adipocyte proteins and a size marker were separated using 8% SDS-PAGE and transferred to a 0.2-μm nylon membrane. The membrane was exposed to Ponceau S stain for 5 min to confirm efficient protein transfer. After 2 h of blocking in 0.5% BSA in Tris-buffered saline with 0.5% Tween (TBST), the membrane was exposed to murine anti–human MCSF monoclonal antibody for 90 min and to secondary antibody for 45 min before exposure using ECL.

To determine the cellular localization of adipocyte MCSF, human subcutaneous adipose tissue was embedded in cryopreservation medium and frozen on dry ice. Immunostaining (9) was conducted on 20-μm-thick frozen tissue sections using murine anti–human MCSF monoclonal antibody incubated at 4°C for 3 h. Controls in the absence of primary antibody were included.

Identification of human MCSF receptor gene expression by adipocytes

Subcutaneous adipocytes were isolated from adipose tissue in two female patients undergoing surgery. RNA was extracted in duplicate and treated with DNase as described above. RT was carried out on 200 ng of DNase-treated RNA using random hexamers in the presence and absence of reverse transcriptase. PCR was carried out using 10% of the RT products and using duplicate 2-μl vol of a 1:10,000 dilution of cDNAs obtained from a human adipocyte library (controls without the library cDNAs were included) using two sets of primers specific for human MCSF receptor (c-fms) mRNA (set 1: forward primer, nucleotides 2794–2815, and reverse primer, nucleotides 3223–3247 and set 2: forward primer, nucleotides 1010–1034, and reverse primer, nucleotides 1492–1514 of MCSF receptor mRNA) (GenBank accession number XO3663) for 35 cycles (denaturing 95°C, annealing 63°C, and elongation 72°C; terminal elongation 4 min). The products were separated using agarose gel (1.5%) electrophoresis, stained with ethidium bromide, and photographed using ultraviolet light. The PCR products were used as templates, with the forward primer and the reverse primer, in separate cycle sequencing reactions.

Regulation of adipocyte MCSF expression by TNF-α in vitro

Subcutaneous adipose tissue was obtained from three other healthy human subjects and the adipocytes isolated as described above. Duplicate aliquots of the adipocytes were suspended in DME at 37°C for 3 h in the presence of variable concentrations of human recombinant TNF-α. RNA was isolated as described above and 2.5 μg of total RNA was applied to positively charged membranes using a slot-blot apparatus. The RNA samples from each subject were applied to the same membrane. Each membrane was hybridized for 10 h at 65°C with the antisense, psoralen-labeled RNA probe to human MCSF mRNA. After development and exposure to x-ray film, the probe was stripped by immersing the membrane twice in 0.1% SDS in diethyl pyrocarbonate water for 20 min at 121°C in an autoclave. The membrane was redeveloped and exposed to x-ray film to ensure that the probe was effectively stripped. The membrane was then hybridized for 4 h at 65°C with an antisense, psoralen-labeled RNA probe to 28S ribosomal RNA, developed, and exposed to x-ray film. Optical densitometry was performed on the x-ray films obtained after hybridization with the MCSF and 28S ribosomal RNA probes. MCSF gene expression was defined as the intensity of the signal obtained using the MCSF probe divided by the intensity of the signal obtained using the 28S probe. MCSF sense and RNA controls in serial dilutions, as well as RNA-diluent controls, were included.

Expression of human adipocyte MCSF in mesenteric fat

Mesenteric fat was obtained from two patients undergoing surgery for inflammatory bowel disease. In each case there was gross evidence of bowel inflammation and close adherence to it of adipose tissue that appeared to have grown in direct relationship to the site of inflammation. In each case adipose tissue was collected from a site adjacent to visibly inflamed bowel and from a region of mesenteric fat connected to normal-appearing bowel > 30 cm distant to the site of inflammation. Adipocytes were isolated and Northern blot analysis for MCSF mRNA (15 μg) was performed. The MCSF probe was stripped from the membrane and the membrane was hybridized with the antisense RNA probe for human 28S ribosomal RNA as described above. Cellular extracts were prepared from 10^7 cells in 500 μl of water, as described above, and 6 μl of the extract was applied to a nitrocellulose membrane. The membrane was stained with Ponceau S, blocked in 0.5% BSA, and exposed to the murine anti–human MCSF monoclonal antibody as described above.

Physiological regulation of human adipocyte MCSF expression

The dietary requirements necessary to maintain steady-state body weight were established over 2 wk in six nonobese volunteers (three women and three men; 58 ± 1 [SE] kg). The subjects were then overfed over 8 wk so as to gain 3–4 kg in fat mass (4–7 kg in weight). Body fat mass was measured before and after overfeeding in duplicate using dual x-ray absorptiometry. Subcutaneous abdominal fat was biopsied (10) before and after overfeeding and adipocyte RNA was isolated as described above. Differential MCSF gene expression was measured before and after weight gain using RNA slot blots (2.5 μg total RNA/slot) as described above. RNA samples from the six subjects, as well as positive and negative controls, were applied to a single membrane. Optical densitometry results obtained with the probe for MCSF mRNA were expressed relative to 28S ribosomal RNA controls as described above. RNA samples were applied to a second membrane (2.5 μg total RNA/slot) to ascertain differential lipoprotein lipase (LPL) expression. This membrane was hybridized with an antisense RNA probe for LPL mRNA (base pairs: 1812–2542; American Type Culture Collection clone 95696; GenBank accession number M15856) for 8 h at 65°C. Optical densitometry results obtained with the antisense RNA probe for LPL were expressed relative to 28S ribosomal RNA controls as described above. Mean adipocyte diameter was estimated by staining $\sim 10^6$ adipocytes in PBS/glucose and methylene blue (5 μg/ml) for 5 min and measuring the cell diameter of 150 cells using light microscopy and a calibrated scale. Human studies were approved by the Mayo Institutional Review Board.

Adenoviral-mediated gene transfer of MCSF to rabbit adipose tissue in vivo

Generation of recombinant adenoviruses. Human MCSF cDNA was subcloned into pMAL123, which contains 355 bp of the left end of adenovirus type 5, 241 bp of the major late promoter, 172 bp from the tripartite leader sequence, a cloning polylinker, an SV40 polyA site, and adenovirus DNA sequences 3330–5790. pMAL123 is a modification of pMCV2 obtained by inserting three linkers at EcoRl, XhoI–BglII, and BglII sites (11). The resulting plasmid was linearized and cotransfected with XbaI-restricted adenovirus strain dl3O9 DNA into 293 cells by conventional calcium phosphate precipitation. Recombinant vectors were generated by homologous recombination. Plaques containing recombinant adenovirus vectors were picked and viral DNA was isolated by a modified Hirt extraction (12). Plaques were screened by restriction mapping and virus was amplified in 293 cells from plaques which contained inserts of the appropriate size. Positive plaques underwent two more rounds of plaque purification. Plaques were again picked and screened by restriction endonuclease mapping. The resulting recombinant adenovirus vectors were expanded in 293 cells and purified by double cesium gradient ultracentrifugation as previously described (11, 13). The isolated viral band was dialyzed against 140 mM NaCl, 10 mM Hepes (pH 7.2), 1 mM $MgCl_2$ and stored at −70°C in l0% glycerol. Human umbilical vein

endothelial cells and 293 cells transduced with MCSF adenovirus (Ad-MCSF) expressed human MCSF which was detected using RT-PCR and Western blot (data not shown). An adenoviral vector encoding β-galactosidase (Ad-βgal) under the control of the CMV promoter was used as a control. Viral titers were determined by plaque assay.

Procedure. Six male New Zealand White rabbits (3.6±0.1 kg) were anaesthetized using 10 mg Acepromazine, 60 mg Rompun, and 300 mg Ketamine. A 3–4-cm midline abdominal incision was made. Ad-βgal and Ad-MCSF were each injected in four injections of 25 μl (2.5×10^{10} pfu) under direct vision into the subcutaneous fat between pairs of sutures separated by 20 mm in either flank of the animals. The two (20-mm-long) regions of subcutaneous fat (one injected with Ad-βgal and the other with Ad-MCSF) were separated by 10 mm and their relative orientation randomly allocated. 10 d after administration of the adenoviral vectors, the animals were killed and the skin and subcutaneous fat flanked by the two sets of sutures was removed in two 20 × 20 mm blocks. The protocol was approved by the Mayo Institutional Animal Care Committee.

Detection of gene transfer. Transfer of the β-galactosidase gene to adipose tissue was detected by positive blue staining of adipocytes with 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (Xgal). Adipocytes from the Ad-MCSF– and Ad-βgal–transduced adipose tissue were isolated as described above. Cells were fixed in a solution of paraformaldehyde (2%) and glutaraldehyde (0.2%) for 10 min and stained in the presence of Xgal for 90 min (14). Transduction efficiency was determined by counting the number of blue-stained cells in a sample of 100 adipocytes using light microscopy.

Transfer of human MCSF using Ad-MCSF was detected using RT-PCR, DNA sequencing, and immunoblotting. Total adipocyte RNA was extracted from the Ad-MCSF– and Ad-βgal–transduced adipose tissue and treated with DNase as described above. RT-PCR

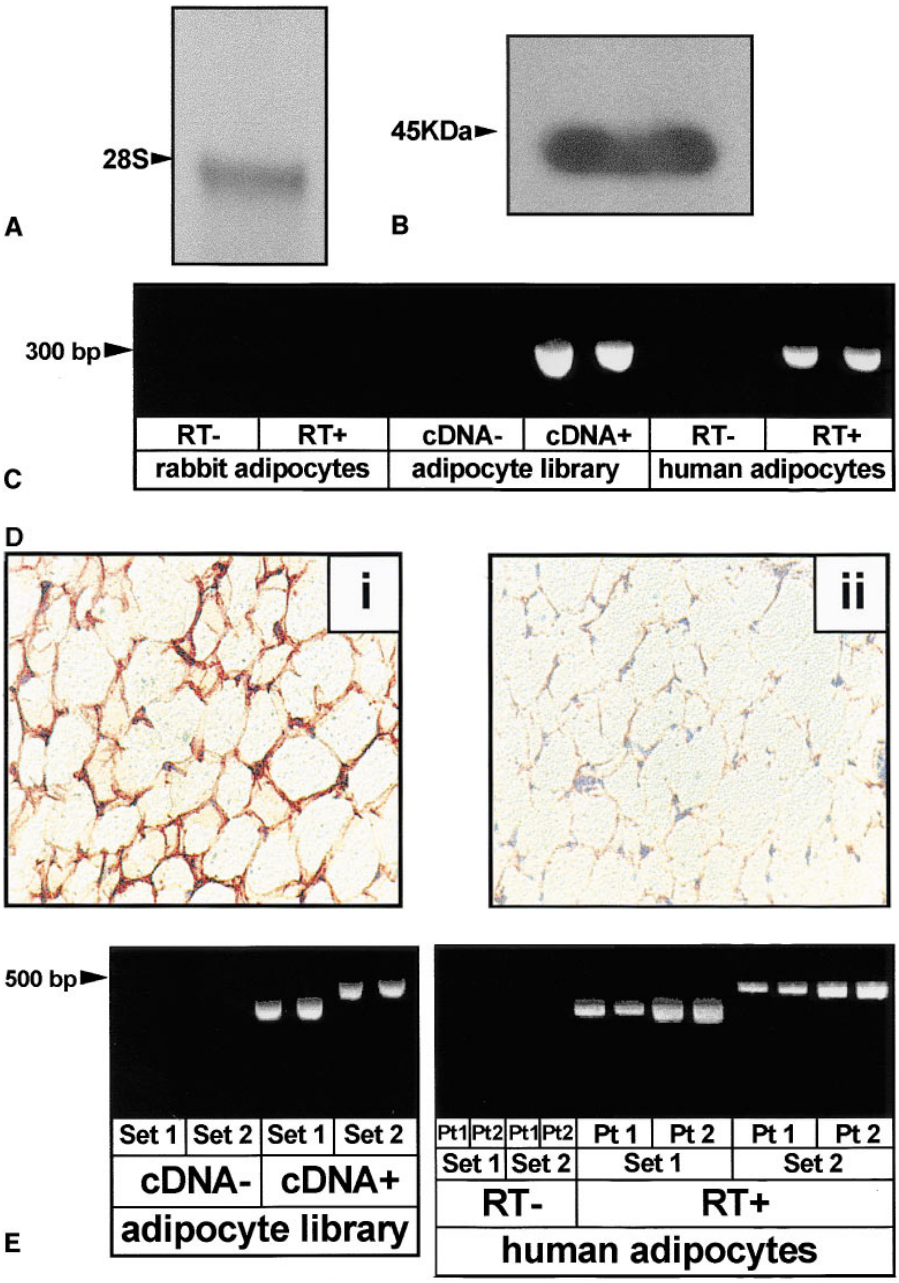

Figure 1. Expression of MCSF and MCSF receptor in adipocytes. (*A*) Northern and (*B*) Western blots for human MCSF. Total RNA and protein were extracted from human adipocytes. RNA was separated using agarose gel electrophoresis and Northern blotting was performed using an antisense RNA probe for MCSF. Adipocyte proteins were separated using gradient layer PAGE and Western blotting was performed using a monoclonal antibody to MCSF. (*C*) RT-PCR for MCSF mRNA in human and rabbit adipocytes and PCR for MCSF cDNA in a human adipocyte library. RT was performed on duplicate aliquots of DNase-treated RNA from human and rabbit adipocytes in the presence and absence of reverse transcriptase (*RT+* and *RT−*); PCR was carried out on the products of these reactions using primers specific for human MCSF mRNA. PCR was also carried out using the same primers and duplicate aliquots of cDNAs from a commercial adipocyte library (*cDNA+*); negative controls without cDNAs (*cDNA−*) were included. (*D*) Immunostaining for human MCSF in adipose tissue in the (*i*) presence of and (*ii*) absence of monoclonal antibody to human MCSF. Human adipose tissue was embedded in cryopreservation medium and sectioned, and immunocytochemistry was performed in the presence and absence of MCSF monoclonal antibody. (*E*) RT-PCR for MCSF receptor mRNA in human adipocytes and PCR for MCSF receptor cDNA in a human adipocyte library. RT was performed in duplicate, on aliquots of DNase-treated RNA from human adipocytes isolated from two patients (*Pt1* and *Pt2*) in the presence and absence of reverse transcriptase (*RT+* and *RT−*); PCR was carried out on the products of these reactions using two sets of primers for human MCSF receptor mRNA (*Set 1* and *Set 2*). PCR was also carried out using the same primers (*Set 1* and *Set 2*) on duplicate aliquots of cDNAs from a commercial adipocyte library (*cDNA+*); negative controls without cDNAs (*cDNA−*) were included.

was performed using the primers specific for human MCSF mRNA as described above; controls without reverse transcriptase were included. PCR products were stained using ethidium bromide, separated using 1.5% agarose gel electrophoresis, and visualized using ultraviolet light. The PCR products were used as templates, along with the forward primer, in cycle sequencing reactions. Protein was isolated from 10^6 adipocytes, in 200 μl of water, using three freeze-thaw cycles, sonication, and lipid extraction. 6 μl of the homogenate was applied to a nitrocellulose membrane, stained using Ponceau S, and incubated with anti–human MCSF mAb for 90 min, secondary antibody for 30 min, and ECL reagents as described above.

Detection of biological effect. The 20 × 20 mm tissue squares were weighed and cut into several pieces. A piece of each block (200–800 mg) was used for quantitative lipid extraction (15). A separate piece was embedded in cryopreservation fluid as described above, sectioned (20 μm), and immunostained with a nuclear marker of proliferation (MIB-1) (16). Nuclear staining was quantified by counting the number of positively staining nuclei out of 100. Independent assessment of nuclear staining by two observers was in agreement by three nuclei. Further sections were prepared for adipocyte sizing; the diameter of 100 cells was measured using light microscopy with a calibrated scale and the mean diameter calculated (17). Any remaining tissue was immersed for 7 d in 20 ml of 10% formaldehyde solution, embedded, sectioned, and stained with hematoxylin and eosin.

To confirm that lipid-rich Ad-MCSF–transduced tissue comprised adipocytes, lipid-rich cells (assumed to be adipocytes) were isolated and cellular protein was isolated in triplicate as described above. Immunoblotting was conducted by applying 2 μl of homogenate to a nitrocellulose membrane and exposing the membrane to murine anti–rabbit Glut4 antibody for 6 h and secondary antibody for 60 min as described above. Positive controls (rabbit femoral adipocytes) and negative controls (rabbit brain) were included. Positive immunostaining was detected using ECL.

Materials

Collagenase, nuclease-free BSA, diethyl pyrocarbonate, paraformaldehyde, and glutaraldehyde were supplied by Sigma Chemical Co. (St. Louis, MO). Glucose, methylene blue, and SDS were supplied by United States Biochemical (Cleveland, OH). PBS was supplied by Celox (Hopkins, MN). The homogenizer was supplied by Brinkman (Westbury, NY). Chloroform was supplied by Curtin Matheson Scientific (Houston, TX). RNAeasy columns were supplied by Qiagen (Santa Clara, CA). Positively charged nylon membranes for Northern and slot blot analyses, psoralen-biotin labeling reagents, psoralen-biotin detection reagents, and the antisense RNA probe for human 28S ribosomal RNA were supplied by Ambion (Austin, TX). Reverse transcriptase (M-MLV) was supplied by Promega (Madison, WI). The human adipocyte library was supplied by CLONTECH (Palo Alto, CA). Cycle sequencing reagents and *Taq*-polymerase were supplied by Perkin-Elmer (Norwalk, CT). Ponceau S stain and protein size marker were supplied by BioRad (Hercules, CA). Acrylamide (30%) was supplied by National Diagnostics (Atlanta, GA). Nylon membranes (Protran 0.2 μm) for Western and immunoblotting and the slot-blot apparatus were supplied by Schleicher & Schuell (Dassel, Germany). Murine anti–human MCSF mAb (MAB216) was supplied by R & D Systems (Minneapolis, MN) and murine anti–rabbit GLUT4 antibody (GLUT4abmx) was supplied by Research Diagnostics Inc. (Flanders, NJ). Secondary antibody was supplied by Transduction Laboratories (Lexington, KY). ECL reagents were supplied by Amersham (Buckinghamshire, England). The cryopreservation medium was supplied by International Equipment Co. (Needham, MA). Recombinant human TNF-α was supplied by R & D Systems. X-ray film (X-OMAT) was supplied by Eastman-Kodak (Rochester, NY). The software for optical densitometry was NIH image supplied by the National Institutes of Health (Bethesda, MD). The weighing scale for human studies was supplied by Scale-Tronix (Wheaton, IL). The dual x-ray absorptiometry scanner (QDR4500) was supplied by Hologic (Waltham, MA). Acepromazine was supplied by Vedco Inc. (St. Joseph, MO). Rompun was supplied by Bayer Corp. (Shawnee Mission, KS). Ketamine was supplied by Fort Dodge Laboratories (Fort Dodge, IO). Xgal and RNase-free DNase were supplied by Boehringer Mannheim (Indianapolis, IN). The pMAL123 clone was a kind gift of Jeff O'Brian (DuPont Merck Pharmaceuticals, Glenolden, PA) and Ad-βgal was a kind gift of Dr. James Wilson (University of Pennsylvania, Philadelphia, PA).

Statistical analysis

Comparison of variables before and after overfeeding for the six human subjects and between Ad-MCSF– and Ad-βgal–transduced adipose tissue squares were made using paired *t* tests. Statistical significance was defined as $P < 0.05$. Data are expressed as mean±SE.

Results

Identification of MCSF mRNA and protein in human adipocytes. Northern and Western blotting confirmed the presence of appropriately sized MCSF mRNA and protein in human adipocytes (Fig. 1, *A* and *B*). RT-PCR confirmed that the gene product was present in human adipocytes and that the amplified cDNA was specific for human adipocytes, as it was absent in cDNA obtained from rabbit adipocytes (Fig. 1*C*). Also, MCSF cDNA was present in the commercial, human adipocyte library (Fig. 1 *C*). The results of immunostaining demonstrated that MCSF was both membrane-bound and located in the cytoplasm (Fig. 1 *D*).

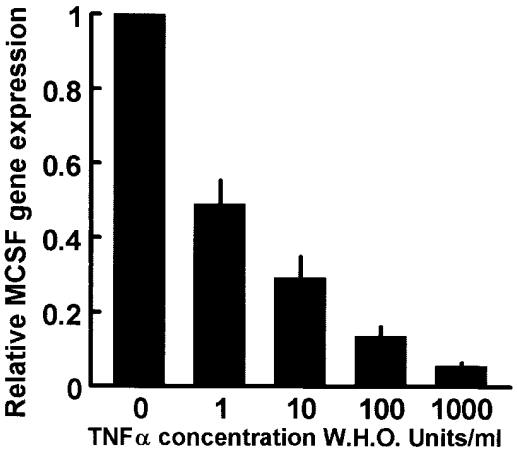

Figure 2. Regulation of adipocyte MCSF gene expression in the presence of varying concentrations of TNF-α in vitro. Isolated human adipocytes were exposed, in duplicate, to varying concentrations of TNF-α in vitro for 3 h. Total RNA was extracted and applied to a nylon membrane using a slot-blot apparatus. The membrane was hybridized with an antisense, psoralen-labeled RNA probe to human MCSF mRNA, developed, and exposed to x-ray film, and the signal intensity of each slot was determined using optical densitometry. The membrane was stripped of the probe and hybridized with an antisense, psoralen-labeled RNA probe to 28S ribosomal RNA, developed, and exposed to x-ray film, and the signal intensity of each slot was determined using optical densitometry. MCSF gene expression was defined as the intensity of the signal obtained using the MCSF probe divided by the intensity of the signal obtained using the 28S probe. Relative MCSF expression, shown on the *y* axis, represents correction of MCSF gene expression to unity which was defined as MCSF expression in the presence of no TNF-α. Data shown represent mean (±SE) values for three patients. 1 World Health Organization unit = 2.5×10^{-5} μg of TNF-α.

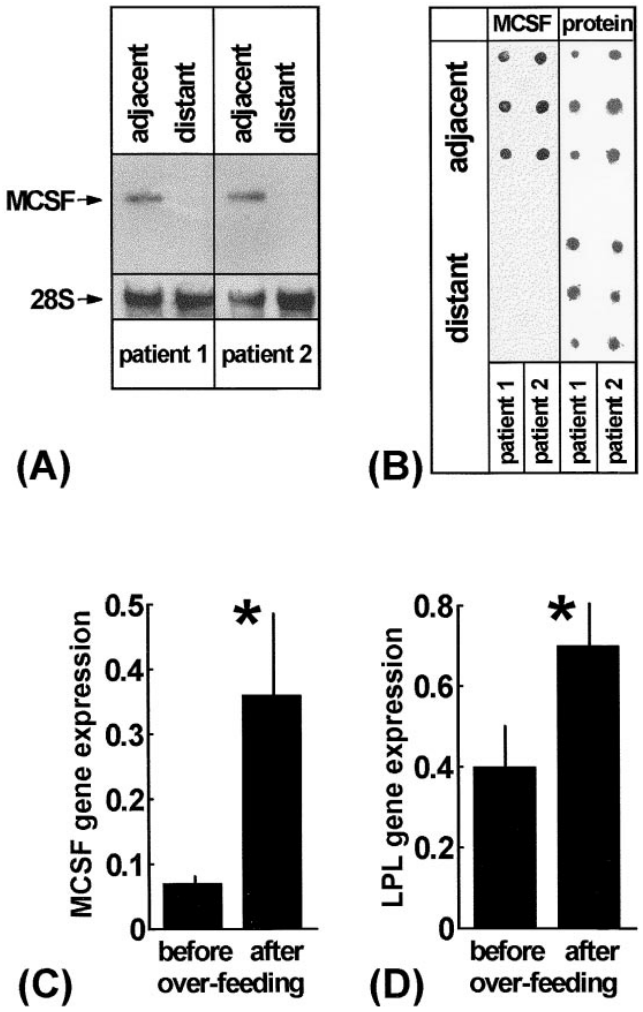

Figure 3. Differential adipocyte MCSF expression in growing human adipose tissue. (*A*) Northern and (*B*) immunoblots for MCSF from mesenteric adipocytes distant from and adjacent to bowel inflammation. Adipocytes were isolated from adipose tissue adjacent to and distant from the site of inflammation in two patients with inflammatory bowel disease. Total RNA was isolated and Northern blots were performed using antisense RNA probes to human MCSF and 28S ribosomal RNA. Whole-cell protein extracts were prepared and immunoblotting was performed in the presence of a monoclonal antibody to MCSF. Efficient protein transfer was demonstrated by staining the membrane with Ponceau S. (*C*) Regulation of adipocyte MCSF and (*D*) LPL gene expression with overfeeding in humans. Six human subjects were overfed so as to gain in excess of 3 kg body fat. Subcutaneous adipocytes were obtained before and after weight gain. Total adipocyte RNA was isolated and applied to a nylon membrane using a slot-blot apparatus. The membrane was hybridized with an antisense, psoralen-labeled RNA probe to human MCSF mRNA, developed, and exposed to x-ray film, and the signal intensity of each slot was determined using optical densitometry. The membrane was stripped of the probe and hybridized with an antisense, psoralen-labeled RNA probe to 28S ribosomal RNA, developed, and exposed to x-ray film, and the signal intensity of each slot was determined using optical densitometry. MCSF gene expression was defined as the intensity of the signal obtained using the MCSF probe divided by the intensity of the signal obtained using the 28S probe. RNA samples were applied to a second membrane and hybridized with an antisense, psoralen-labeled RNA probe to human LPL mRNA and the 28S RNA probe as before. LPL gene expression was defined as the intensity of the signal obtained using the LPL probe divided by the intensity of the signal obtained using the 28S probe. MCSF and LPL gene expression were compared for the six subjects before and after weight gain and statistical significance was defined as $*P < 0.05$.

Identification of MCSF receptor mRNA in human adipocytes and adipocyte library. RT-PCR and cycle-sequencing confirmed that human adipocytes express MCSF receptor mRNA (c-fms) (Fig. 1 *E*). Also, MCSF receptor cDNA was present in the commercial, human adipocyte library (Fig. 1 *E*).

Regulation of adipocyte MCSF expression by TNF-α in vitro. TNF-α has been implicated as a mediator of adipocyte lipid accumulation (18–20), and in cultured adipocytes TNF-α inhibits adipocyte differentiation which in turn is associated with downregulation of MCSF gene transcription (7). Exposure of human adipocytes to varying concentrations of TNF-α in vitro was associated with dose-dependent downregulation of human adipocyte MCSF gene expression (Fig. 2).

Differential expression of human adipocyte MCSF in mesenteric adipose tissue that grows around inflamed bowel. MCSF gene and protein expression were upregulated in the human adipocytes isolated from the adipose tissue adjacent to inflamed bowel compared with adipocytes isolated from adipose tissue > 30 cm away from the site of inflammation (Fig. 3, *A* and *B*).

Regulation of human adipocyte MCSF expression in response to overfeeding. The coefficient of variation for repeat measurements of MCSF gene expression using sense MCSF standards was < 3%. The mean weight of the six subjects before overfeeding was 58±1 kg and increased to 64±1 kg with overfeeding. Body fat increased by a mean of 3.6±0.2 kg. Adipocyte MCSF gene expression increased from 0.07±0.01 to 0.36±0.13 arbitrary units ($P < 0.05$) (Fig. 3 *C*). Adipocyte LPL gene expression was upregulated with overfeeding from 0.4±0.1 to 0.7±0.1 arbitrary units ($P < 0.05$) (Fig. 3 *D*). Mean adipocyte diameter did not increase significantly (69±5 to 72±3 μm).

Adenoviral-mediated gene transfer of MCSF to rabbit adipose tissue in vivo. All animals tolerated the gene transfer procedures well. Successful transfer of β-galactosidase was detected in the Ad-βgal–transduced subcutaneous adipose tissue in each animal. The mean number of adipocytes staining blue with Xgal was 10±1% of cells. Adenoviral-mediated gene transfer appeared highly localized, as whole tissue cross-sections exposed to Xgal failed to show staining of the dermis or epidermis. No blue-staining cells were detected in adipocytes isolated from the Ad-MCSF–transduced adipocytes. RNA isolated from the adipocytes transduced with Ad-MCSF yielded an RT-PCR product of the expected molecular weight (292 bp) using the primers specific for human MCSF mRNA. The PCR product was confirmed to be human MCSF mRNA by DNA sequencing in each animal. There were no PCR products of the correct molecular weight amplified from the adipocytes exposed to Ad-βgal. Immunoblotting confirmed that adipocytes exposed to Ad-MCSF expressed human MCSF protein and that the adipocytes exposed to Ad-βgal did not (Fig. 4).

Overexpression of MCSF in the subcutaneous adipose tissue was associated with visible increases in subcutaneous fat in all six animals which did not occur in the subcutaneous fat transduced by Ad-βgal (Fig. 5 *A*). This was consistent with 3-fold (range 2.0–3.2) increases in mass of the 20 × 20 mm tis-

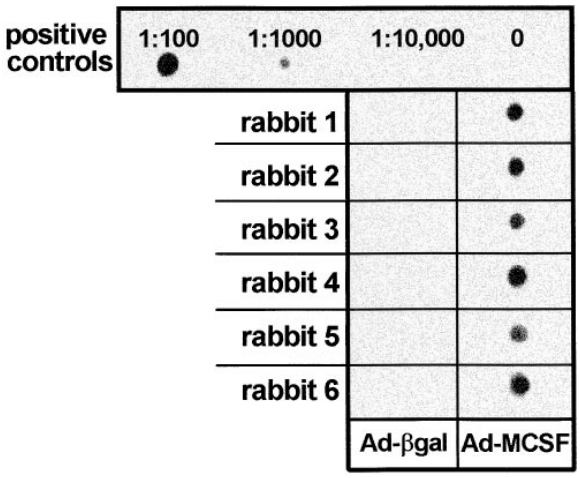

Figure 4. Detection of human MCSF protein in rabbit subcutaneous adipocytes transduced with Ad-βgal and Ad-MCSF in vivo. Six New Zealand White rabbits were anesthetized and a midline abdominal incision was made. Under direct vision, aliquots of Ad-βgal and Ad-MCSF were injected into subcutaneous fat between pairs of sutures separated by 20 mm. After 10 d, the animals were killed and the tissue surrounding the pairs of sutures was dissected from the animal in two 20 × 20 mm blocks, one block having been transfected with Ad-βgal and the other with Ad-MCSF. Adipocytes were isolated from these blocks and whole-cell protein was isolated. Immunoblots using a monoclonal antibody specific for human MCSF were performed. Positive controls of recombinant human MCSF were included.

sue squares and 16-fold increase in absolute lipid content (range 7–22) compared with the Ad-βgal controls (Fig. 5, *B* and *C*). Immunostaining with a nuclear marker of proliferation (MIB-1) was increased in the regions of Ad-MCSF–transduced adipose tissue compared with the Ad-βgal controls 13±1% c.f. 3±1% ($P < 0.0005$) (Fig. 5 *D*). The histologic features of the Ad-MCSF–transfected tissue were compatible with those of adipose tissue (Fig. 5 *E*) without consistent or extensive inflammatory infiltrate nor evidence of adipocyte cell division. Protein isolated from these cells stained positively with the Glut4 antibody (Fig. 5 *F*). Mean adipocyte diameter was not increased significantly for adipocytes exposed to Ad-MCSF compared with Ad-βgal (55±6 vs. 51±6 μm).

Discussion

The profound paracrine effects that MCSF exerts on recruitment and terminal differentiation of pluripotent cells in other tissues were reminiscent to us of how adipose tissue grows. Therefore, MCSF was investigated as a potential mediator of adipose tissue hyperplasia. In this paper we demonstrate that: (*a*) adipocytes synthesize MCSF; (*b*) both pathologic and physiologic human adipose tissue accumulation are accompanied by upregulation of adipocyte MCSF; (*c*) TNF-α, a known inhibitor of adipocyte hyperplasia in vitro, inhibits human adipocyte MCSF expression; and (*d*) adenoviral-mediated overexpression of MCSF in vivo results in significant adipose tissue growth through hyperplasia.

The expression of MCSF mRNA and protein by human adipocytes was demonstrated by Northern and Western analyses. Adipocyte MCSF expression was then assessed in models of rapidly growing human adipose tissue where hyperplasia is assumed to occur. An example of fast-growing adipose tissue is thought to be "creeping fat" (21) where mesenteric adipose tissue grows apposed to acutely inflamed bowel. Adipocytes isolated from creeping fat showed upregulation of MCSF expression compared with mesenteric fat distant to the site of bowel inflammation. Although little is known about the process by which adipose tissue appears to envelop inflamed bowel, the localized upregulation of adipocyte MCSF is consistent with our hypothesis that MCSF mediates adipose tissue hyperplasia. The upregulation of human adipocyte MCSF that accompanied adipose tissue deposition with overfeeding is also consistent with the proposed role of adipocyte MCSF on adipose tissue hyperplasia in vivo. The fact that adipocyte MCSF expression was downregulated by TNF-α in vitro represents further supportive evidence that MCSF may participate in adipocyte physiology. In cell culture, TNF-α is known to inhibit adipocyte differentiation in association with downregulation of MCSF (7) and in other tissues TNF-α directly modulates the proliferative effects of MCSF (22, 23). Thus, we demonstrate several lines of evidence to suggest that MCSF participates in adipose tissue hyperplasia. However, these data do not demonstrate that MCSF directly stimulates adipose tissue growth.

Therefore, the direct effect of MCSF on adipose tissue growth was investigated. Although a naturally occurring mouse knock-out of MCSF exists, the *op/op* mouse, it is not applicable to studies of adipose tissue growth. The phenotype of *op/op* mice includes decreased body weight (24–27), absent teeth (24, 25), and altered masticator muscles (28), which account for some, but not all, of the decreased body weight (29). In addition, these animals exhibit impaired mammary gland development with a decreased ratio of adipose-to-glandular tissue (30), abnormal lipid metabolism (26), altered estrogen and androgen regulation (31, 32), decreased pulmonary cellularity (27), and osteopetrosis (24, 25). Although observations from *op/op* mice might be consistent with the putative role of MCSF in adipose tissue growth, confounding variables prohibit meaningful studies of nutritional manipulation in these animals. It was necessary to explore an alternative approach to investigate whether MCSF directly mediates adipose tissue hyperplasia.

Direct injection of MCSF into adipose tissue beds was not feasible because MCSF would be cleared from adipose tissue too rapidly for adipose tissue growth to occur. Therefore, we induced localized, sustained overexpression of MCSF in rabbit subcutaneous adipose tissue using adenoviral-mediated gene transfer that has proven a reliable, efficient method for inducing highly localized gene transfer in other settings (11, 33, 34). We found that localized overexpression of MCSF in the subcutaneous adipose tissue was associated with increased tissue mass. Quantitative lipid extraction demonstrated that the increase in mass was attributable to lipid accumulation. Histology and Glut4 immunoblotting confirmed the tissue to be adipose tissue. The marked increase in adipose tissue clearly resulted from hyperplasia because mean adipocyte size did not increase significantly and immunostaining with a nuclear marker of proliferation (16) was increased in the regions of MCSF-associated adipose tissue accumulation. Since there was no cytological evidence of cell division, we were not able to challenge the tenet that adipocytes are terminally differentiated and do not divide. The substantially greater adipose tissue accumulation in the Ad-MCSF region compared with the con-

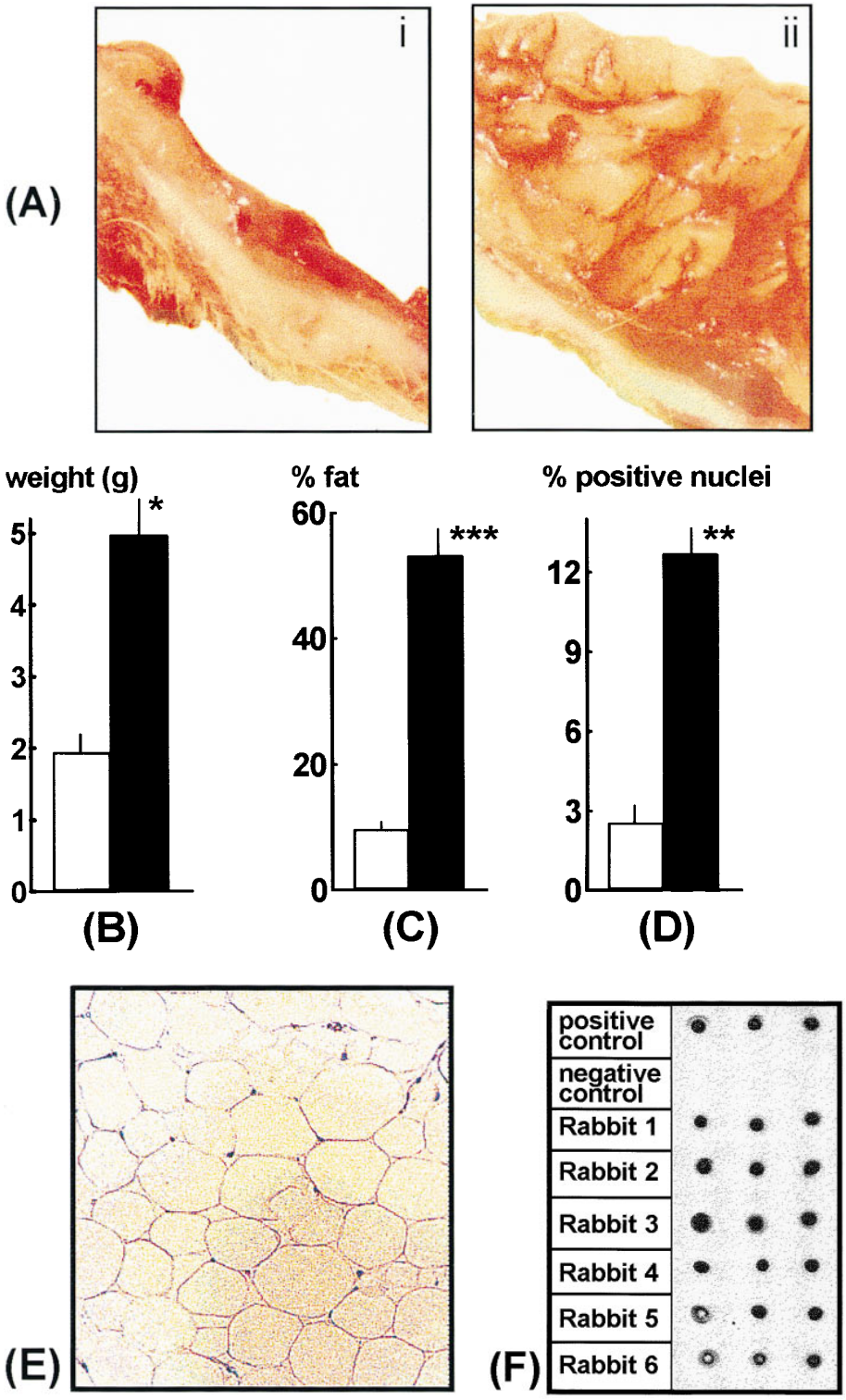

Figure 5. Biological effect of overexpression of MCSF in rabbit subcutaneous adipocytes transduced with Ad-βgal and Ad-MCSF in vivo. Six New Zealand White rabbits were anesthetized and a midline abdominal incision was made. Under direct vision, aliquots of Ad-βgal and Ad-MCSF were injected into subcutaneous fat between pairs of sutures separated by 20 mm. After 10 d, the animals were killed and the tissue surrounding the pairs of sutures was dissected from the animal in two 20 × 20 mm blocks, one block having been transfected with Ad-βgal and the other with Ad-MCSF. (*A*) Representative cross-sectional appearance of tissue blocks from one animal 10 d after transduction with (*i*) Ad-βgal and (*ii*) Ad-MCSF; (*B*) Mass of the 20 × 20 mm squares of skin–subcutaneous tissue (*white bars* in *B–D*, Ad-βgal; *black bars* in *B–D*, Ad-MCSF); (*C*) Lipid content (% fat) of the 20 × 20 mm squares of skin–subcutaneous tissue; (*D*) Percentage of adipocyte nuclei showing positive staining with monoclonal antibody MIB-1; (*E*) Representative hematoxylin and eosin–stained section of tissue block from one animal 10 d after transduction with Ad-MCSF; (*F*) Glut4 immunoblots of Ad-MCSF–transduced tissue. Lipid-rich cells were separated from the Ad-MCSF–transduced tissue and immunoblots were performed, in triplicate, on whole-cell isolates using an antibody specific for the intracellular domain of Glut4. Positive controls (rabbit femoral adipocytes) and negative controls (rabbit brain) were included. Statistical significance was defined as $^{*}P < 0.005$, $^{**}P < 0.0005$, $^{***}P < 0.0001$.

trol virus region demonstrates that this response was not caused by viral infection per se.

We propose that the mechanism by which MCSF mediates adipose tissue hyperplasia is comparable to the effects of MCSF in other cell systems, particularly those where c-fms expression occurs concurrently (35). Furthermore, we speculate that adipocyte TNF-α (19, 20), in part, modulates the effects of MCSF, as we demonstrated in adipocytes in vitro and as has been reported elsewhere (22, 23). Thus, during positive energy balance adipocyte hypertrophy occurs initially. Hypertrophy may be accompanied by or, in part, mediated by TNF-α (18) which inhibits MCSF expression. Once adipocytes exceed a certain size, the process of hyperplasia is initiated whereby MCSF is released and promotes the recruitment of pluripotent precursor cells. MCSF is unlikely to be the sole mediator of adipocyte hyperplasia as it is not tissue specific; MCSF is synthe-

sized by and mediates proliferation in several cell types. It is thus proposed that, perhaps via activation of the c-fms receptor, an adipocyte-specific signal is released that mediates transformation of the recruited pluripotent precursor cells to preadipocytes which then become available for hypertrophy and lipid storage.

In conclusion, MCSF is the first identified factor that actively promotes human adipose tissue hyperplasia. It is synthesized by human adipocytes, and it is upregulated under conditions in which adipose tissue growth is pathologically and physiologically accelerated and MCSF expression is downregulated by TNF-α which is a known inhibitor of adipocyte proliferation. Finally, localized overexpression of adipocyte MCSF using adenoviral-mediated gene transfer resulted in marked, rapid adipose tissue growth through hyperplasia. Thus, if humans overfeed and adipocyte MCSF becomes upregulated and if MCSF overexpression results in profound local adipose tissue hyperplasia, it is reasonable to conclude that MCSF, at least in part, mediates adipose tissue hyperplasia and contributes to adipose tissue deposition in humans. The proximity of the chromosomal locus for human MCSF (1p21) to sites linked with obesity and insulin action (36) heightens the potential importance of MCSF in both the biology of adipose tissue growth and the pathogenesis of obesity.

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