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Adipogenesis inhibitory factor

A novel inhibitory regulator of adipose conversion in bone marrow

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Recombinant adipogenesis inhibitory factor (AGIF) was purified to homogeneity from the conditioned medium of COS-1 cells transfected with human AGIF cDNA. The amino-terminal sequence analysis of the mature AGIF revealed that AGIF was produced as a precursor consisting of 199 amino acids and processed into a mature form of 178 amino acids by a cleavage between Ala(−1) and Pro(+1). The purified AGIF inhibited the process of adipogenesis in mouse 3T3-L1 preadipocytes, indicating that AGIF directly acts on the cells. AGIF acted as an adipogenic antagonist not only on the extramedullary cell line 3T3-L1 but also on the mouse bone marrow stroma-derived cell line H-1/A, suggesting that this cytokine may regulate adipogenesis in bone marrow.

Adipogenesis inhibitory factor; Interleukin-11; Adipocyte differentiation; Lipoprotein lipase; Bone marrow stromal cell

1. INTRODUCTION

Adipocytes are among the major constituents of the bone marrow stroma in adult mammals [1,2]. In vitro studies using long-term bone marrow culture techniques have shown that adipocytes and preadipocytes play some active roles in stromal cell-associated hematopoiesis, and adipogenesis in the bone marrow stroma is believed to affect hematopoiesis in the bone marrow microenvironment [1,3–5]. However, little is known about the regulation of adipogenesis in the bone marrow.

We recently reported the isolation from a human bone marrow-derived stromal cell line of a cDNA encoding a novel adipogenesis inhibitory factor (AGIF) and identity with interleukin-11 (IL-11) [6,7]. Conditioned medium of COS-1 cells transfected with an AGIF expression plasmid suppressed lipoprotein lipase (LPL) in 3T3-L1 adipocytes [6]. However, it remains to be determined whether suppression of LPL in the cells is due to a direct action of AGIF. In this report, we describe the purification of AGIF from the transfected COS-1-cell conditioned medium, determination of the amino-terminal sequence of mature AGIF, and biological activities of purified, mature AGIF.

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2. MATERIALS AND METHODS

2.1. *Construction of AGIF expression plasmid*

The expression plasmid pSRα-20-2 was constructed by replacing the stuffer region of pcDL-SRα296 [8] with the cDNA from pcD-20-2 [6] that encodes a human AGIF precursor. COS-1 cells were transfected with pSRα-20-2 according to the DEAE-dextran protocols with chloroquine treatment as described [9] and the serum-free conditioned medium was obtained.

2.2. *Purification of recombinant AGIF*

Serum-free conditioned medium (7.5 liters) of transfected COS-1 cells was dialyzed against 10 mM borate, pH 9.0, containing 13 mM KCl (designated hereafter as 'borate buffer'). The dialyzed sample was applied to a CM-Toyopearl 650M column (2.2 × 20.2 cm; Tosoh, Tokyo, Japan) and eluted with a 0–0.3 M linear gradient of sodium chloride in the borate buffer. Fractions were subjected to an LPL assay and to Western blotting. The material from the CM-Toyopearl column was equilibrated with borate buffer plus 0.5 M sodium chloride and loaded onto a Superdex 75pg column (2.6 × 60 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden). Marker proteins (Bio-Rad Laboratories, Richmond, CA) were run on the gel filtration column under the same conditions. The concentrated material from Superdex 75pg was equilibrated with the borate buffer plus 20% ammonium sulfate, applied to a Phenyl-Toyopearl 650M column (2.2 × 10 cm; Tosoh), and eluted with a 20–0% linear gradient of ammonium sulfate in the borate buffer. Protein concentrations were determined by use of Bio-Rad Protein Assay Kit (Bio-Rad Labs.) or Micro BCA Protein Assay Reagent (Pierce, Rockford, IL).

2.3. *Western blotting*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [10]. Western blotting was conducted by use of the Immun-Blot Assay Kit (Bio-Rad Labs.). Rabbit antisera were raised against a synthetic AGIF peptide that corresponds to the 27th to 41st amino-acid residues of the human AGIF precursor deduced from a cloned cDNA [6]. The Ig fraction

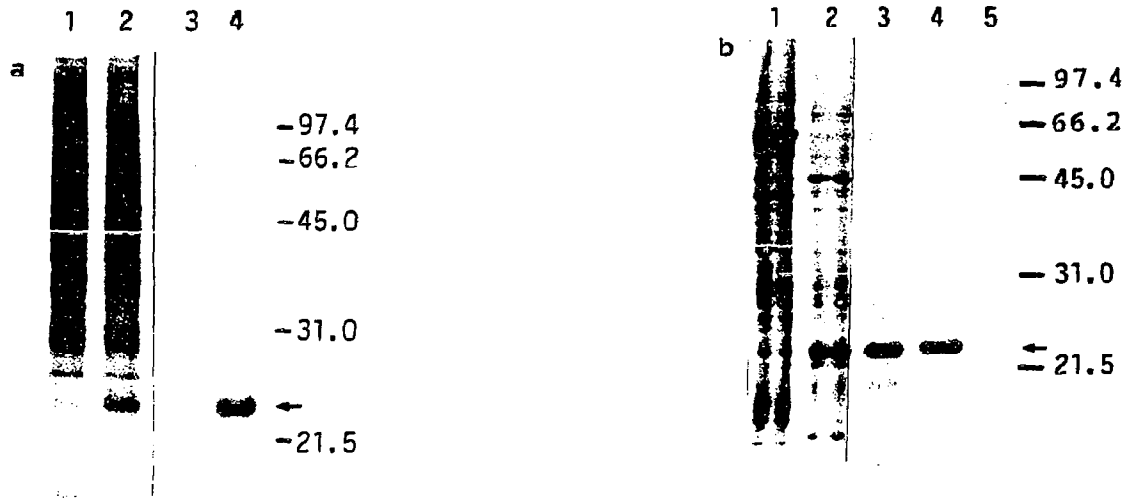

Fig. 1. (a) SDS-PAGE and Western blot analysis of AGIF protein secreted by transfected COS-1 cells. Serum-free culture supernatant (160 μl) from COS-1 cells transfected with a negative control plasmid, pcDL-SRα296 (lane 1), or with pSRα-20-2 (lane 2) was concentrated by trichloroacetic acid precipitation and analyzed under reducing conditions on a 12.5% polyacrylamide gel followed by silver staining. The pcDL-SRα296 or pSRα-20-2 transfection supernatant was further subjected to Western blot analysis (lanes 3 and 4, respectively). (b) Analysis of purified, mature AGIF by SDS-PAGE. Aliquots of the samples in each purification step were analyzed under reducing conditions on a 12.5% polyacrylamide gel followed by silver staining. Samples: culture supernatant of COS-1 cells transfected with pSRα-20-2 (lane 1); materials from CM-Toyopearl (lane 2), Superdex (lane 3) and Phenyl-Toyopearl (lane 4); and sample buffer only (lane 5). The relative positions of standard proteins (low-molecular-weight standards, Bio-Rad Labs.) analyzed in parallel are as indicated (masses in kDa). The positions of mature AGIF are shown by arrows.

(designated as anti-AGIFp15) was prepared from the antisera and used for Western blotting.

2.4. *Amino acid sequence analysis*

AGIF-containing fractions from CM-Toyopearl were pooled, electrophoresed on a 12.5% SDS-polyacrylamide gel, and transferred electrophoretically to an Immobilon membrane (Millipore, Bedford, MA). The area corresponding to the band of AGIF was cut out for protein microsequencing. Amino-terminal sequence determination was carried out on a gas-phase protein sequencer (model 475A, Applied Biosystems, Foster City, CA).

2.5. *AGIF biological assay*

LPL-suppression activity was determined as described in our previous report [6]. To estimate inhibition of adipose conversion in 3T3-L1 cells by AGIF, we treated cultures of the cells that had reached confluence for 4 days with adipocyte-induction medium (complete DMEM [6] containing 5 μg of insulin per ml, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine) supplemented with various concentrations of purified AGIF. The medium was then replaced with maintenance medium (complete DMEM containing 100 ng of insulin per ml) supplemented with AGIF at the same concentrations as above, and the cells were cultured for an additional 4 days. Next, the cultures were fixed with 5% formaldehyde, and the nuclei and the lipid droplets that had accumulated in the cells were then stained with hematoxylin and Oil red O, respectively. Photographs were taken, and the percentage of adipocytes was calculated from the number of nuclei (stained light-purple) and adipocytes that contained red-stained lipid vacuoles. H-1/A cells [11] were grown at 33°C in Fischer medium containing 10% FBS. After the cells had reached confluence, the medium was replaced with the adipocyte-induction medium (Fischer medium containing 10% FBS and 1 μM hydrocortisone) supplemented with several dilutions of conditioned medium of COS-1 cells transfected with pSRα-20-2, and the cells were cultured for 20 days. During the culture period, the medium was exchanged every 2–5 days for fresh induction medium containing the COS-1 conditioned medium. The cells were then fixed and stained, and the percentage of adipocytes was determined.

3. RESULTS

3.1. *Purification of recombinant AGIF*

To characterize the protein encoded by the AGIF cDNA, we constructed an expression plasmid, pSRα-20-2, that directs a high level expression of the

Table 1

Purification of recombinant human AGIF

Step	Volume (ml)	Total protein (μg)	Total activity (U × 10^{-3})	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Conditioned medium	7,500	122,000	600	4.9×10^3	1	100
CM-Toyopearl 650M	17.7	920	195	2.1×10^5	43	32
Superdex 75pg	14.8	74	16	2.2×10^5	45	2.7
Phenyl-Toyopearl 650M	20.2	19	9	4.9×10^5	100	1.5

One unit (U) of AGIF is defined as the amount of factor required to induce a 50% suppression of LPL activity in 3T3-L1 adipocytes cultured in a volume of one ml.

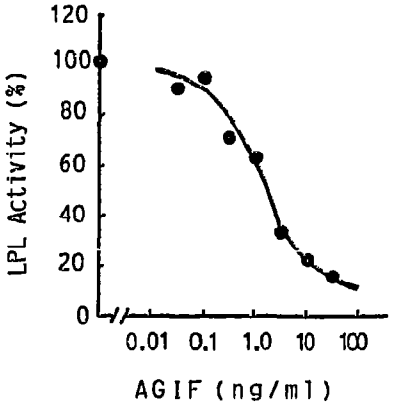

Fig. 2. Dose-response of LPL activity in AGIF-treated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were exposed to the indicated concentrations of purified AGIF for 18 h, after which heparin-released LPL activity was determined. LPL activity was expressed relative to that in untreated cultures, in which more than 80% of the cells had accumulated lipid droplets, and which was defined as 100%.

cDNA in COS-1 cells. Western blot analysis by use of anti-AGIFp15 antibodies confirmed that the protein encoded by the cDNA was secreted in the conditioned medium of transfected COS-1 cells, and the molecular mass of the secreted AGIF was estimated to be 23 kDa (Fig. 1a).

The purification scheme consisted of the following steps: (i) dialysis of conditioned medium against borate buffer; (ii) ion-exchange chromatography; (iii) size-exclusion chromatography; and (iv) hydrophobic-interaction chromatography. In each purification step, AGIF was monitored by its LPL-suppression activity in 3T3-L1 adipocytes and by Western blot analysis. In all purification steps, fractions that exhibited LPL-suppression activities corresponded to those showing AGIF bands in Western blotting. Table I summarizes the results of the procedures used to purify recombinant AGIF. Size-exclusion chromatography resulted in a symmetrical peak of LPL suppression activity in the fractions corresponding to apparent molecular masses of ~17 kDa, indicating that AGIF may be present as a monomer (data not shown). The Phenyl-Toyopearl fractions that had exhibited LPL suppression activities yielded a single protein peak migrating as a 23-kDa band when analyzed by SDS-PAGE under reducing conditions (Fig. 1b), and this protein was identified as AGIF by Western blotting (data not shown). The final specific activity was estimated to be 4.9×10^5 U/mg protein.

3.2. *Amino-terminal sequence of mature AGIF*

We determined the sequence of the first ten amino-terminal residues of mature AGIF as NH_2-Pro-Gly-Pro-Pro-Pro-Gly-Pro-Pro-Arg-Val-. This result indicates that AGIF is synthesized as a precursor of 199 amino acids and processed into its mature form by a cleavage between the Ala (−1) and Pro(+1) residues, which results in a protein of 178 amino acids with a predicted molecular weight of 19 144.

3.3. *Biological activity of AGIF*

Purified AGIF suppressed the heparin-releasable LPL activity of differentiated 3T3-L1 adipocytes in a dose-dependent manner (Fig. 2). This effect was evident at concentrations as low as 0.5 ng/ml and was maximal at doses of more than 10 ng/ml. This result indicates that AGIF directly acts on 3T3-L1 adipocytes. Moreover, AGIF inhibited the process of adipogenesis in 3T3-L1 cells in a dose-dependent manner (Fig. 3). The effective doses were almost the same as in the case of LPL suppression in the 3T3-L1 adipocytes.

We also tested the effect of AGIF on adipogenesis in the mouse bone marrow stroma-derived cell line H-1/A, which is derived from the adherent cell layer of a 14-week-old Dexter bone-marrow culture and thought to have originated from adventitial reticular cells in the bone marrow [11]. AGIF inhibited the adipogenic differentiation of H-1/A cells (Fig. 4) as well as it did that of 3T3-L1 cells.

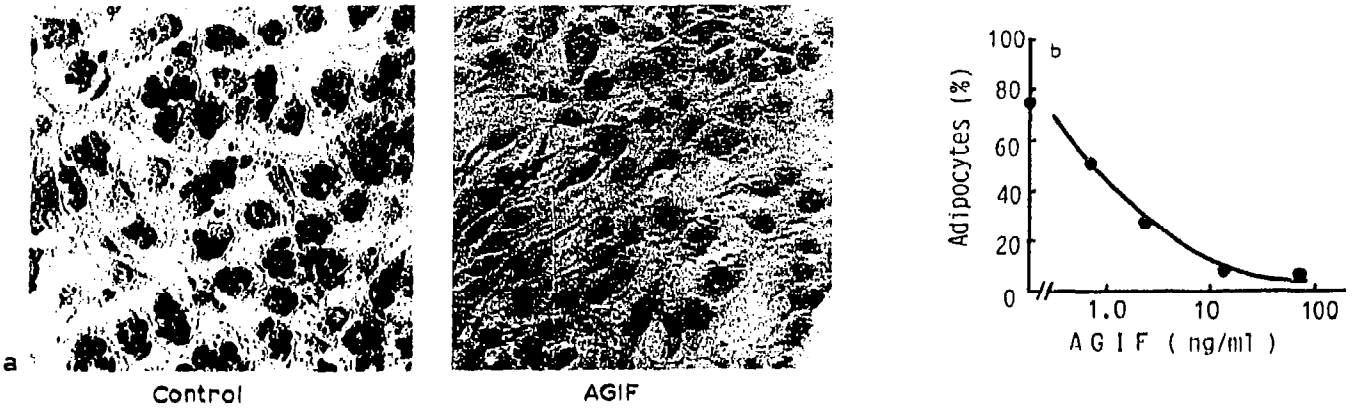

Fig. 3. Inhibition of adepogenic differentiation of 3T3-L1 cells by AGIF: (a) photographs (×150); (b) percentage of adipose conversion. 3T3-L1 cells were induced to differentiate into adipocytes in the presence of the indicated concentrations of purified AGIF. Photographs depict the results in the presence and absence (indicated as control) of AGIF (12.5 ng/ml).

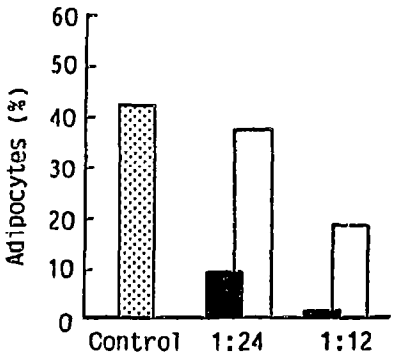

Fig. 4. Inhibition of adipogenesis in the bone marrow stroma-derived cell line H-1/A by AGIF. H-1/A cells were cultured for 20 days in adipocyte-induction medium supplemented with the indicated final dilutions of conditioned medium of COS-1 cells transfected with pcDL-SRα296 (a negative-control plasmid) or pSRα-20-2. Control means no addition of COS-1 conditioned medium. Open bars and closed bars indicate pcDL-SRα296 and pSRα-20-2, respectively.

4. DISCUSSION

We have purified mature AGIF from medium conditioned by transfected COS-1 cells and tested its ability to act as an antagonist of adipogenesis in H-1/A cells and 3T3-L1 cells. Amino-terminal sequence analysis of mature AGIF indicates that this protein consists of 178 amino acids with a predicted molecular weight of 19 144. The apparent molecular mass (23 kDa) estimated by SDS-PAGE analysis (Fig. 1) is larger than this calculated molecular mass (19 kDa). Since AGIF lacks potential N-linked glycosylation sites and was eluted with the fractions corresponding to apparent molecular masses of ~17 kDa on a size-exclusion column, the smaller mobility of AGIF on SDS-polyacrylamide gels might be due to the strongly basic characteristics of this protein (calculated pI = 11.3). We cannot, however, rule out the possibility of any post-translational modification(s).

AGIF directly suppressed the heparin-releasable LPL in 3T3-L1 adipocytes (Fig. 2), but it did not affect the activity of the enzyme itself (data not shown). Therefore, LPL-suppression by AGIF may be mediated by its inhibitory action in any step(s) of the LPL-biosynthesis pathway in the cells. AGIF is expressed in the bone marrow-derived stromal cell line KM-102 [6], and this stroma-derived factor inhibited the process of adipogenesis in the bone marrow stroma-derived H-1/A preadipocytes (Fig. 4). H-1/A cells produce colony stimulating factors, and the colony stimulating activity of the cells decreases during their adipocyte differentiation [4,5]. Another preadipose cell line, PA6, which is thought to originate from bone marrow preadipocytes, has a unique capacity to support the growth of CFU-S through a cell-to-cell interaction [12]. PA6 cells fail to interact with hematopoietic cells and their growth-supporting activity for CFU-S decreases when the cells differentiate into adipocytes [3]. We therefore hypothesize that AGIF is an inhibitory regulator of adipose conversion in bone marrow and plays a role in keeping stromal preadipocytes hematopoietically active by inhibiting their adipogenic differentiation. The AGIF cDNA is now known to be identical to that of IL-11, a cytokine recently reported to be a hematopoietic factor for B cells and megakaryocytes [7,13]. AGIF/IL-11 may be a multifunctional factor that acts on both stromal cells and hematopoietic progenitor cells in the bone marrow microenvironment.

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