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Research Article

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Rapid Publication

Release of Mitogenic Factors by Cultured Preadipocytes from Massively Obese Human Subjects

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Abstract

In this study, possible paracrine factors in adipose tissue from lean and obese subjects were sought. Conditioned media were prepared by incubation in alpha minimum essential medium of adipocyte precursors derived from lean and massively obese subjects. Adipocyte-precursor–derived conditioned media from the obese stimulated replication of cultured rat perirenal adipocyte precursors by about fourfold over control. The effect of media conditioned by precursors derived from lean subjects was much less evident. The mitogenicity of conditioned media was abolished by trypsin, indicating the protein nature of the mitogenic factor(s). Sephacryl S-200 chromatography of adipocyte-precursor–derived conditioned media from obese subjects revealed one major active fraction with molecular masses in the range of 25,000–40,000. Our results demonstrate that adipocyte precursors derived from massively obese subjects release factors mitogenic on cultured rat adipocyte precursors. These principles may act as paracrine factors contributing to the development of the adipocyte hyperplasia characteristic of massive obesity.

Introduction

We have previously reported that adipocyte precursors derived from massively obese (both childhood- and adult-onset) subjects replicate excessively in culture (1, 2). This in vitro phenomenon may result from an inherent aberration in genetic expression in the precursor cells. This concept is supported by the fact that excessive proliferation was observed in both cloned adipocyte precursors and cells in successive subcultures (2). Furthermore, when mature human fat cells are induced to revert in culture to a fibroblastlike form, those from the massively obese not only regain the ability to replicate but in fact proliferate more rapidly than similarly treated cells from lean persons (3). This genetic expression may be mediated by release of factors that act via paracrine mechanisms to stimulate neighboring cells to undergo division. The present study was designed to explore this hypothesis. We examined the mitogenicity of adipocyte-precursor–derived conditioned media from lean and massively obese subjects on rat adipocyte precursors in culture. In this paper, we present evidence for exaggerated release of mitogenic factors from the massively obese and describe the partial characterization of these proteins.

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Methods

Materials. Disposable tissue culture supplies were purchased from Corning Canada Inc., Toronto, Ontario. Alpha minimum essential medium (MEM)[1] with and without nucleosides, Hanks' balanced salt solution (HBSS) powder, fetal bovine serum (FBS), penicillin, and gentamicin were obtained from Gibco, Grand Island, NY. Trypsin, soybean trypsin inhibitor, Na_2EDTA, and ammonium acetate were purchased from Sigma Chemical Co., St. Louis, MO. 2-Mercaptoethanol was obtained from Eastman–Kodak Co., Rochester, NY. Sephacryl gel was obtained from Pharmacia Fine Chemicals, Montreal, Quebec. [Methyl-^{3}H]thymidine (67 Ci/mmol) and Econofluor scintillation fluid were purchased from New England Nuclear, Boston, MA; GF/A glassfibre filters were bought from Whatman Inc., Clifton, NJ.

Preparation of conditioned media from cultured adipocyte precursors. Adipocyte precursors were isolated from human and rat adipose tissue according to our previously reported methods (1–4). Human omental adipose tissue was obtained, following an informed consent, from 6 lean and 10 massively obese subjects undergoing elective abdominal surgery. Massive obesity is operationally defined as body weight exceeding reference values by > 70%. The age, sex, body weight, and body mass index of all 16 subjects are listed in Table I. All precursor cell strains were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Complete alpha MEM supplemented with 10–15% FBS, 15 mM Hepes, penicillin (100 U/ml), and gentamicin (50 μg/ml) was used for all the studies except those described below. For the production of conditioned media, human cells between subcultures 2 and 5 were inoculated into 75-cm^2 culture flasks at a density of 4,000 cells/cm^2 in alpha medium initially supplemented with 15% FBS. Conditioned media were collected at each of the following growth phases: 3 d postinoculation, 3 d before, and at monolayer confluence. 24 h before the collection, cells were washed thrice with HBSS, and fresh alpha medium with 0.5–1% FBS was dispensed to the culture. At the end of each collection period, cells in parallel culture flasks were detached with 5 ml 0.5 mg/ml trypsin–2 mM Na_2EDTA in Na_2-citrate-KCl (pH 7.4) solution, and duplicates of 2-ml aliquots were counted in isotonic counting fluid (Isoton; Coulter Electronics, Inc., Hialeah, FL) using a model Z_f Coulter counter (Coulter Electronics, Inc., Hialeah, FL), as previously reported (2, 3). Conditioned media were filtered through a 0.45-μM filter (Millipore/Continental Water Systems, Bedford, MA), and stored frozen at −70°C until use.

Assay of mitogenicity of conditioned media. Mitogenicity was quantified by stimulation of replication of rat retroperitoneal adipocyte precursors, as assessed by direct cell counting and by incorporation of [methyl-^{3}H]thymidine into trichloroacetic acid–insoluble macromolecules (DNA) (1–3). In this bioassay system, rat adipocyte precursors

1. *Abbreviation used in this paper:* MEM, minimum essential medium.

Table I. Characteristics of Lean and Massively Obese Subjects

Subject no.		Age	Sex	Height*	Weight‡	% > ref§	BMI§
Lean subjects							
1	I.M.	43	M	1.78	75.0	0	23.7
2	R.D.	60	F	1.59	53.0	0	21.0
3	M.N.	45	M	1.75	69.8	0	22.8
4	M.T.	42	F	1.52	52.0	0	22.5
5	J.K.	30	F	1.60	61.1	0	23.8
6	M.T.	52	F	1.65	63.6	0	23.4
Mean±SEM		45.3±4.1		1.65±.04	62.4±3.7	0	22.9±0.4
Massively obese subjects							
7	D.M.	38	F	1.71	134.5	106	46.5
8	E.E.	38	F	1.72	142.0	113	48.0
9	I.M.	47	F	1.76	116.0	70	37.4
10	D.B.	32	F	1.68	110.0	73	39.1
11	R.M.	45	M	1.73	115.9	73	38.7
12	W.S.	55	F	1.58	117.0	106	46.9
13	L.S.	33	F	1.63	118.2	100	44.7
14	V.M.	34	F	1.73	114.0	71	38.1
15	A.N.	24	F	1.59	129.5	145	51.4
16	A.S.	52	F	1.59	102.0	80	40.5
Mean±SEM		39.8±3.1		1.67±.02	119.9±3.8	98.7±7.8	43.1±1.6

Massive obesity is operationally defined as body weight exceeding reference values by >70%. * Height in meters. ‡ Weight in kilograms. § Abbreviations: % > ref, percent of body weight above reference values; BMI, body mass index (wt in kg/[height in m]^2).

from second or third subcultures were inoculated (5×10^4 cells/well) into 35-mm six-well plates and grown in alpha MEM with 10% FBS for 24 h. Fresh alpha MEM (without nucleosides) and 1% FBS were added on day 2 to induce quiescence before the addition of various concentrations (1–50% vol/vol) of conditioned media on day 3. On day 5, cells were washed extensively with phosphate-buffered saline and detached from the wells with 0.5 mg/ml trypsin-2 mM Na_2EDTA in Na_2-citrate-KCl (pH 7.4) solution. Cells were counted in Isoton with the Coulter counter and validated by hemocytometer (1–4). In the case of radioactive thymidine incorporation, one μCi of [methyl-^{3}H]thymidine was added on day 4 to each well, and its incorporation into trichloroacetic-acid–insoluble macromolecules (DNA) was determined 24 h later. The cell suspension was transferred to a sampling manifold fitted with Whatman glassfibre filters. The radioactivity retained on the filters following washing with 10% trichloroacetic acid was counted in a Nuclear-Chicago Corp. (Des Plaines, IL) liquid scintillation spectrophotometer using Econofluor scintillation fluid (1–3, 5).

Effect of trypsin on conditioned media. To test whether the mitogenicity was due to polypeptide factors, conditioned medium was preincubated with 1 mg/ml trypsin at 37°C for 30 min. The conditioned media were incubated for another 30 min with 3 mg/ml soybean trypsin inhibitor before addition to cultured rat adipocyte precursors. To sets of control experiments were included: (*a*) preincubation of conditioned medium with 3 mg/ml trypsin inhibitor for 30 min at 37°C, followed by 1 mg/ml trypsin for 30 min; (*b*) preincubation of conditioned medium only for 60 min at 37°C.

Sephacryl S-200 chromatography of conditioned media. ~ 1 l of media conditioned by precursors derived from massively obese subjects was pooled, concentrated to ~ 4 ml, and applied to a 2.5 × 90–cm Sephacryl S-200 gel exclusion column equilibrated with 0.05 M ammonium acetate (pH 7.0) and 0.05 M 2-mercaptoethanol, at a flow rate of 15 ml/h. The 4.5-ml eluate fractions were arbitrarily pooled together and concentrated using an Amicon apparatus and UM-50 filters (with M_r 3,000 exclusion size). Protein determination was performed with a protein assay kit (Bio-Rad Laboratories, Richmond, CA), which was calibrated against gamma globulin and bovine serum albumin as protein standards (6). The mitogenicity of the pooled and concentrated eluate fractions on rat adipocyte precursors was determined as described above.

Statistical analyses. The results were statistically analyzed using Student's paired *t* test.

Results

Table I summarizes the clinical data on all lean and massively obese subjects. Omental adipose tissue samples were obtained from subjects who were undergoing elective cholecystectomy or, in the case of the massively obese, gastric stapling procedures for massive obesity. The mean age of lean subjects was slightly higher than that of the massively obese subjects.

In assessing the mitogenicity of adipocyte-precursor–derived conditioned media from lean and massively obese persons, correction was made for the greater number of cells present in the cultures derived from the massively obese. Thus, the mitogenic activity was always expressed as a function of the same number of cells in the lean and obese populations. Since quantification of cell replication by both direct cell enumeration and tritiated thymidine incorporation into DNA yielded similar results, only data on the latter were presented in this report. To ensure reproducibility of mitogenicity, at least two experiments were performed on the same adipocyte-precursor–derived conditioned media using different rat retroperitoneal adipocyte precursors in the bioassay system. Thus the data presented herein for each of the 16 adipocyte-precursor–derived conditioned media were the mean from at least two bioassays.

In all experiments, conditioned media derived from adipo-

cyte precursors from the massively obese consistently stimulated the replication of rat adipocyte precursors in culture (Fig. 1). The effect was seen with the addition of as little as 10% and was maximum with the addition of 30% of this conditioned medium (vol/vol). The results presented in Fig. 1 were obtained from experiments on adipocyte-precursor–derived conditioned media collected from lean and massively obese subjects. These results were highly significant with a P value < 0.005 using Student's paired t test. In contrast, conditioned media derived from cultures of cells from lean subjects were much less stimulatory. Out of six experiments stimulation was evident in only four, and the maximal stimulation achieved was 150% above control. However when all data from lean subjects were pooled, there was no statistical difference from controls, even at maximal concentrations.

In the next series of experiments observations were made of the mitogenic activity of conditioned media at various times relative to confluence (Fig. 2). As previously noted, activity in media exposed to cells of obese subjects was much higher than that from lean patients. In all experiments activity was highest in media collected from cells at time of confluence. Since the mitogenicity was compared on the basis of identical number of cells from which the conditioned media were prepared, the highest activity observed at time of confluence may be due to maximal production of mitogens by the precursor cells at this stage or the elaboration of a more potent compound.

The next experiments were designed to determine the nature of the mitogenic activity. Conditioned media from cells of both lean and obese subjects were preincubated with trypsin. As shown in Fig. 3, exposure to trypsin abolished the mitogenic activity of conditioned media derived from cells of massively obese subjects. Preincubation with excess trypsin inhibitor prevented the suppressive influence of trypsin on the mitogenic factors in the conditioned media; trypsin itself did not inhibit precursor replication.

Next, a pool of conditioned medium from precursors of obese subjects was fractionated on Sephacryl S-200. The results of this

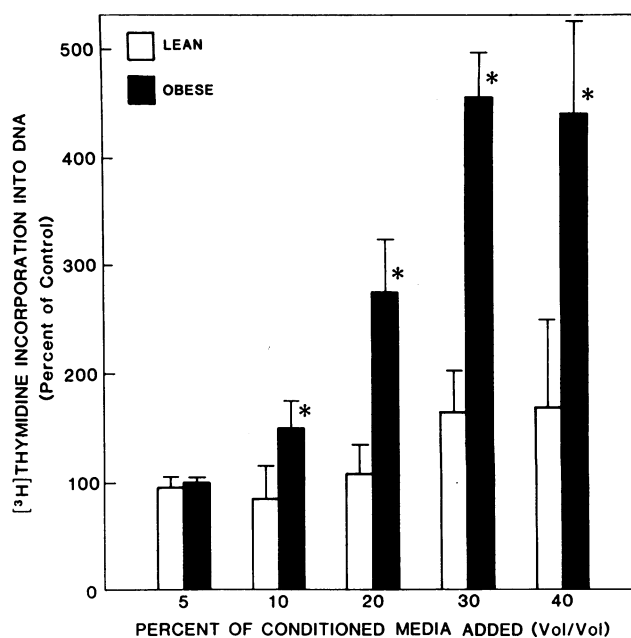

Figure 1. Effects of media conditioned by omental adipocyte precursors from lean and massively obese subjects on incorporation of [^{3}H]thymidine into DNA of cultured rat adipocyte precursors. 20% of added conditioned medium represents 0.4 ml within a total of 2 ml of culture medium used for each assay. Each milliliter of conditioned medium was collected from 4×10^4 cells from the lean or from 4.8×10^4 cells from massively obese subjects. The results were expressed as percent incorporation of [^{3}H]thymidine into DNA of control (without conditioned medium). The data represent the mean±SEM of experiments on adipocyte-precursor–derived conditioned media produced by adipocyte precursors from six lean and 10 massively obese subjects. The difference between conditioned media of cells from lean as compared with massively obese subjects is highly significant at $P < 0.005$ (asterisks) except at 5%. The slight effect at higher concentrations of conditioned media from cells of the lean did not attain statistical significance.

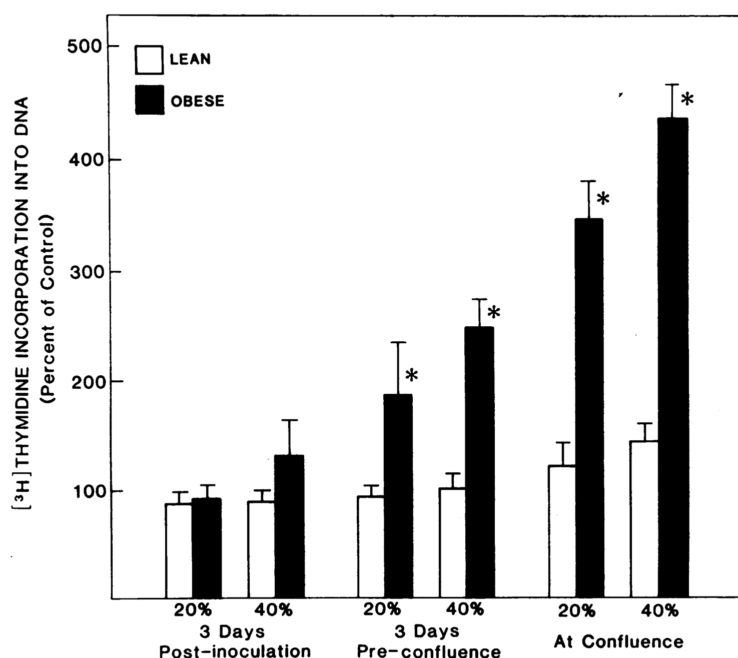

Figure 2. Effect of media conditioned by adipocyte precursors at different stages in culture. Different amounts of conditioned media collected at each growth phase were tested for their mitogenic activity on rat adipocyte precursors. Only the concentrations for 20% and 40% (vol/vol) are shown for each of the three growth phases. The results are the mean±SEM of experiments on conditioned media derived from adipocyte precursors of six lean and 10 massively obese subjects. As before, asterisks denote statistically significance at $P < 0.005$. Other details are described in the legend of Fig. 1.

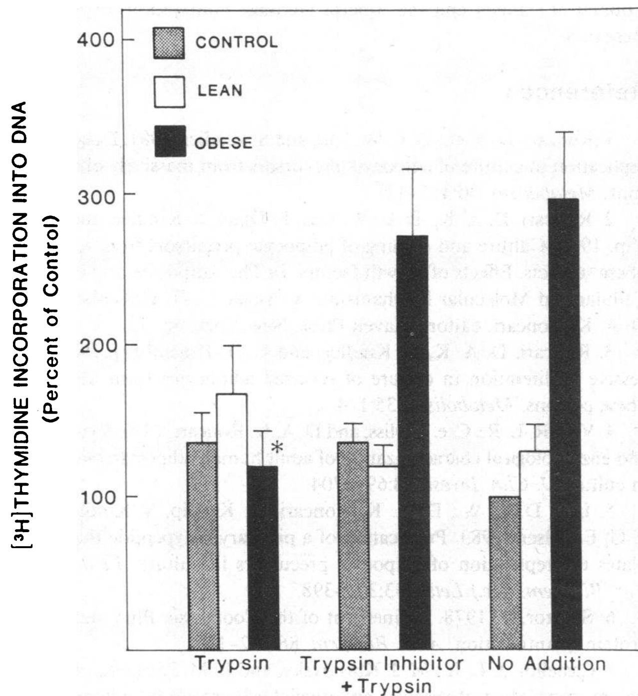

Figure 3. Effect of trypsin on media conditioned by adipocyte precursors derived from lean and massively obese subjects. Conditioned media derived from cells of lean or massively obese subjects were incubated with 1 mg/ml trypsin at 37°C for 30 min, followed by 3 mg/ml soybean trypsin inhibitor, as detailed in Methods. Controls included (*a*) preincubation of conditioned medium with 3 mg/ml soybean trypsin inhibitor followed by 1 mg/ml trypsin; (*b*) incubation of conditioned media only for 60 min. The controls shown in the figure by *shaded bars* were precursor cells incubated without any conditioned media. *Asterisk* indicates statistical significance ($P < 0.05$) between trypsin-treated conditioned media preincubated in the absence or presence of trypsin inhibitor.

study are shown in Fig. 4 and the details of the experiment are described in the legend for this figure. While several broad protein peaks were eluted, mitogenic activity was largely recovered in a pooled fraction containing relatively little protein. The molecular mass for this active fraction, as deduced from molecular size standards used for calibrating the same Sephacryl S-200 column, was in the range of 25,000–40,000 D. Mitogenic activity was also present in fractions corresponding to molecular mass between 40,000 and 67,000 D.

Discussion

This study indicates that cultured adipocyte precursors from massively obese subjects release principle(s) that stimulate the replication of rat adipocyte precursors. Precursors from lean persons apparently release similar principles, but to a considerably lesser extent. Should these mitogenic principles also be released in vivo, they would act as paracrine/autocrine factors that would increase the number of adipocyte precursors. Maturation of these precursor cells to triglyceride-laden adipocytes would consequently lead to expansion of adipose tissue mass. Since release of these trophic principles is particularly prominent by cells of the massively obese, this phenomenon may be of pathophysiologic significance in this marked form of corpulence.

As is the case for other growth factors, the mitogenic principles released by adipose cells apparently comprise a group of related proteins. The most active protein(s) isolated under our conditions from cultured cells of massively obese subjects has a molecular mass of 25,000–40,000 D. The relatively low molecular mass is in keeping with the notion that these mitogens are secretory proteins released by the precursor cells.

Recently, a number of growth factors have been identified in 3T3 fibroblasts that are mitogenic on a variety of cells in culture, including endothelial and epithelial cells (7). Whether the mitogen(s) described herein are similar to those reported

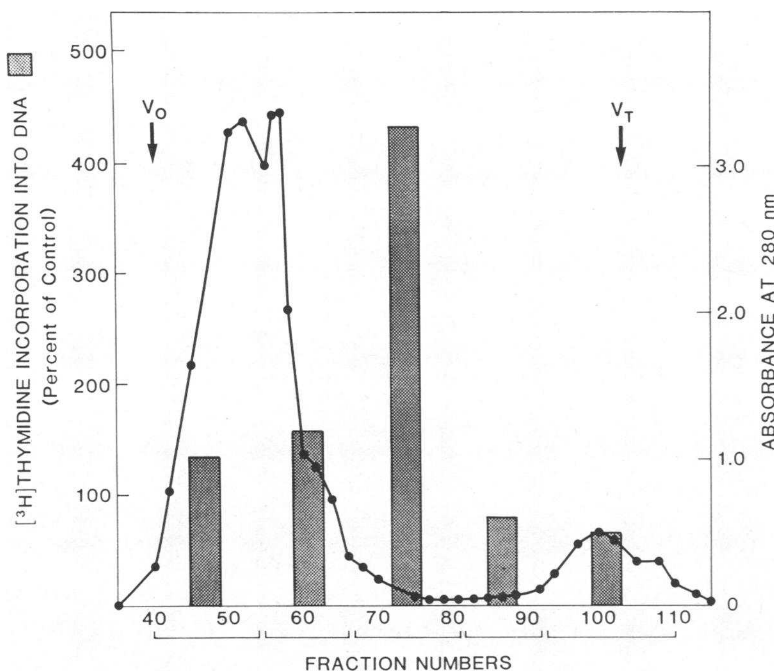

Figure 4. Gel filtration chromatography of media conditioned by adipocyte precursors derived from massively obese subjects. A 2 × 90–cm Sephacryl S-200 column was equilibrated in 0.05 M ammonium acetate (pH 7.0) and 0.05 M 2-mercaptoethanol. The protein profile is indicated by absorbance (O.D.) at 280 nm. V_0 and V_t denote void volume and total elution volume, respectively. The eluate fractions were arbitrarily pooled as indicated by the square brackets. The degree of incorporation of [^{3}H]thymidine into DNA by each concentrated fraction is represented by the *shaded bars*. The most active fraction corresponded to a molecular mass of 25,000–40,000 D, as estimated by molecular size standards.

remains to be determined. Although we tested the adipocyte-precursor–derived conditioned media on adipocyte precursors only, it would not be surprising if the conditioned media were also mitogenic on other cells of mesenchymal origin. Normal fetal-derived human lung fibroblasts (WI-38) release into the culture medium factors that cross-react by radioimmuno- and radioreceptor assays with insulinlike growth factor I (8). We are currently exploring whether these are related to the mitogenic proteins released by adipocyte precursors.

Growth regulation and regeneration of normal tissues are dependent on intercellular (endocrine and neural) and cell-to-cell communication, including the paracrine/autocrine mechanism (9). Through this mechanism, it is possible that the proteins described in this paper act as progression factors inducing cells in quiescence to enter G_1 from G_0 phase of the cell cycle (10). Should these factors be produced and secreted in vivo in adipose tissue of massively obese persons, exaggerated replication of adipocyte precursors would be produced, which in turn would lead over a number of cycles, to an expanded pool of these potential adipocytes. Coupling of exaggerated replication of precursors with promotion of maturation would eventually produce a greater complement of adipocytes. Further, excessive production of trophic paracrine/autocrine factors could confer partial autonomy to adipose tissue in massive obesity, promoting progressive expansion relatively independently of stimuli external to fat depots.

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