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TRANSFORMATION OF OB17 CELLS PROMOTES PROLIFERATION AND DIFFERENTIATION OF OB17 PREADIPOCYTES VIA DISTINCT EXTRACELLULAR INTERMEDIATES

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Conditioned serum-free medium of Ob17 cells transformed by the middle-T-only gene of polyoma virus (Ob17MT cells) is able to support growth and adipose conversion of the parental Ob17 cells. Conditioned media from 3T3-F442A cells (untransformed preadipocyte clonal line) and MTT4 cells (middle-T-transformed non-preadipocyte clonal line) are inactive. The serum-free conditioned medium of Ob17MT cells is also active on growth and adipose conversion of 3T3-F442A cells. The morphological differentiation of Ob17 cells is accompanied by the expression of early (lipoprotein lipase, LPL) and late (glycerol-3-phosphate dehydrogenase, GPDH) biochemical markers of adipose conversion. Bio-Gel P-60 chromatography and SDS-PAGE have allowed characterization of a mitogenic fraction of apparent MW $\simeq$ 28 K_d distinct from an adipogenic fraction of apparent MW <10 K_d. This adipogenic fraction is only required for the acquisition of the GPDH activity and is therefore active on terminal differentiation.

Ob17 cells transformed by the middle-T-only gene of polyoma virus (Ob17MT cells) have been shown to differentiate in vitro and in vivo into adipose cells. In vitro, the serum requirement for growth was diminished and the hormonal requirements for differentiation were simpler than those of the original Ob17 cells : the adipose conversion could take place in serum-free, triiodothyronine-, growth hormone-supplemented medium (1). These reduced requirements for growth and differentiation might well be related, at least in part, with the secretion of growth factors of the transforming growth factor type. These factors active on growth could be or not the same as those active on differentiation. Preliminary investigations led us to observe that conditioned media of Ob17MT cells were able to support growth and differentiation of the original Ob17 cells. This observation prompted us to characterize the extracellular factor(s) involved in the process of adipose conversion since we had previously reported that Ob17 cells were able to differentiate in a serum-free medium containing growth hormone, triiodothyronine, insulin, fetuin and a low-molecular weight com-

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ponent(s) purified from serum (2). In addition, we have shown recently that the adipose conversion of Ob17 cells and related subclones involves the expression of at least two separate sets of markers (early and late) which are regulated differently by hormones (3-5). This observation prompted us also to characterize the stage of adipose conversion at which these extracellular factors might be involved. The results reported below show that Ob17MT cells secrete a factor(s) active on growth which can be distinguished from a factor(s) active upon adipose terminal differentiation.

MATERIALS AND METHODS

Clonal lines

The characterization of clonal lines Ob1771, Ob17MT15, Ob17MT18 and Ob17PY has been previously described (1,6). Clonal lines MDCK (7), MTT4 (8) and 3T3-F442A (9) were also previously characterized.

Conditioned media

Conditioned media were obtained after seeding 10^4 cells in 100-mm dishes and growth of the different clones in DME medium supplemented with 3% (clones Ob17MT15, Ob17MT18 and Ob17PY) or 10% FBS (clones MDCK, MTT4 and 3T3-F442A) as previously described (1). All cultures were incubated at 37°C under an atmosphere of 95% air:5% CO_2. At confluence (approximately 5 days after seeding), cultures were rinsed four times (30 min each) with a mixture of DME : Ham's F12 medium (1:1 ; v/v) containing 15 mM $NaHCO_3$, 15 mM Hepes buffer, pH 7.4, 33 µM biotin, 17 µM panthothenate, 62 mg/ml penicillin, 50 mg/l streptomycin, 5 mg/l insulin and 5 mg/l transferrin. This medium is referred to as serum-free medium. After a 24 hour incubation in this medium, the latter was discarded and replaced by fresh medium. Two subsequent 48-hour collections were taken ; both were pooled and this medium is referred to as initial conditioned medium. It was first dialyzed extensively at 4°C against 0.1 M acetic acid (Spectrapor tubing with an exclusion limit of 3,500 daltons) and then centrifuged at 10,000 g for 30 min. The supernatant was saved and lyophilized. The lyophilizate was solubilized with 0.1 M acetic acid, under conditions corresponding to a 100-fold concentration with respect to the initial conditioned medium. This solution is now referred to as conditioned medium.

Fractionation of conditioned media

Conditioned medium from MDCK cultures (2 ml) or from Ob17MT15 and Ob17MT18 cultures (14 ml) were chromatographed on 150-ml or 1000-ml Bio-Gel P-60 columns, respectively, after equilibration with 0.1 M acetic acid. Elution was performed in both cases at a flow rate of 20 ml/hour and 3-ml fractions were collected. The pooled fractions I to V (see Fig.1) were lyophilized. Each lyophilizate was solubilized in a volume of 0.1 M acetic acid identical to that loaded on the column and stored at -20°C until use. All subsequent experiments of cell growth and differentiation, using conditioned media or derived fractions, were performed on Ob1771 cells. The samples were analyzed on a 14-20% acrylamide gel according to the method of Laemmli (10) and visualized using a sensitive silver stain method (11), with or without prior treatment in the presence of 2-mercaptoethanol.

DNA synthesis and cell growth

[^{3}H]thymidine incorporation into DNA was determined as previously described (12). Cell growth was determined by cell enumeration with a Coulter counter after seeding Ob1771 cells in 24-well plates (5000 cells per well) in 1 ml of DME medium containing 10% FBS. Twenty-four hours later, cells were rinsed and maintained in serum-free medium supplemented or not with 1.7 nM EGF and either conditioned medium or fractions to be assayed. Media were renewed 3 days after inoculation, as previously described (2). Cell enumeration was performed 6 days after inoculation using the Giemsa staining-elution procedure (13).

Cell differentiation and enzyme assays

After growth in serum-free medium supplemented with conditioned medium as described above (12-well plates ; 2 ml per 24 mm-well), confluent Ob1771 cells (day 0) were exposed for the indicated periods of time to serum-free medium supplemented with 0.15 nM T_3, 1.4 nM b-GH and either conditioned medium or the different fractions. This medium, defined as differentiation serum-free medium, was supplemented only between day 0 and day 6 with 0.1 mM IBMX. Media were changed every 6 days. When required, cells were rinsed with a PBS solution at 37°C, then resuspended and homogeneized in 20 mM Tris-Cl buffer, pH 7.4 containing 150 mM NaCl. GPDH and LDH activities were determined directly on cell homogenates. LPL was assayed after complete disruption of membrane structures by adding Triton X-114, as previously described (14). Enzyme activities are expressed in nmol/min per mg of protein (15).

RESULTS

As shown in Table I, conditioned medium (CM) of Ob17MT18 cells, in the presence of insulin and transferrin, was able to support growth of Ob1771 cells (a non-transformed sub-clone of Ob17 cells), as determined by DNA synthesis and cell enumeration. In the absence of CM, cells remained viable but mitoses did not take place significantly. The same CM was also able to support cell differentiation of Ob1771 cells, as shown by micrographs of Figure 1Bb and by quantitative assays of LPL and GPDH activities (Table I) as representative of early and late markers of adipose conversion, respectively (2). This observation was not unique to Ob1771 preadipocytes :

Table I **Growth and differentiation of Ob1771 cells in conditioned media from clonal lines**

ADDITION	DNA SYNTHESIS cpm/well 10^{-3}	CELL GROWTH (fold increase)	CELL DIFFERENTIATION			
			LIPOPROTEIN LIPASE		GLYCEROL-3-PHOSPHATE DEHYDROGENASE	
			−	+	−	+
None	5.4	1.0	*	*	*	*
CM - Ob17MT15	38.7	3.1	2.0	7.2	und.	1100
CM - Ob17MT18	63	2.55	1.7	3.5	und.	464 (146) [und.]
CM - Ob17PY	28.6	2.33	-	-	und.	395
CM - MDCK	54.4	2.35	2.3	8.8	2.1	465 (376) [und.]
CM - MTT4	7.2	*	*	*	*	*
CM - 3T3-F442A	8.2	*	*	*	*	*

DNA synthesis and cell growth (expressed as in Fig.1) were determined after addition of 5 µl of each 100-fold concentrated conditioned medium (**CM**) in 0.5 ml of serum-free medium. LPL and GPDH activities were determined 16 days post-confluence, after addition of 20 µl of the same conditioned medium used for growth in 2 ml of serum-free medium supplemented (+) or not (-) with IBMX, T_3 and GH as described in the "Materials and Methods" section. und., undetectable. The results are representative of 4, 4, 1, 3, 1 and 1 experiment(s) using conditioned medium from Ob17MT15, Ob17MT18, Ob17PY, MDCK, MTT4 and 3T3-F442A cells, respectively. The numbers in parentheses and square brackets correspond to the values obtained with 3T3-F442A and 3T3-C_2 cells, respectively. Asterisks indicate a loss of cell viability.

it could be extended to 3T3-F442A preadipocytes which differentiate into adipose cells (9) but not to 3T3-C2 cells which are unable to do so (9) (Table I). Conditioned medium of Ob17MT15 cells, a clone showing a high potentiality to overproliferate at low serum concentration and a low potentiality to differentiate (1), also supported growth and differentiation of Ob1771 cells. Conditioned medium of Ob17PY cells, which were obtained after transfer of the complete early region of polyoma virus (1), showed similar properties to those of conditioned media from Ob17MT18 and Ob17MT15 cells. The ability to support growth and differentiation of Ob1771 cells did not seem to be specific to conditioned media from cells established from mouse and having a mesodermal origin since growth and differentiation of Ob1771 and 3T3-F442A cells took place in the presence of conditioned medium from MDCK cells, an epithelial cell line established from dog kidney. In contrast to conditioned media from Ob17MT18, Ob17MT15 and MDCK cell lines, conditioned medium from 3T3 cells which were established from Fischer rat embryo (17) and subsequently transformed by middle-T (8), as well as conditioned medium from untransformed 3T3-F442A cells which were established from mouse embryo (9), were inactive. These two latter conditioned media were not only unable to support growth and differentiation of Ob1771 cells but actually led to a loss of cell viability. No conditioned medium could be obtained from Ob1771 cells since the cells lost viability under the serum-free conditions used for Ob17MT cells and detach from the substratum within 24 hours. The fractionation of conditioned medium from Ob17MT18 cells on Bio-Gel P-60 column, equilibrated with 0.1 M acetic acid, is shown in Figure 1. Four peaks were detected. Among the five fractions assayed for cell growth, Ob1771 cells were able to reach confluence in the presence of fractions I, II and III only. Fraction III showed the highest mitogenic activity, whether or not the culture medium of Ob1771 cells was supplemented with an optimal concentration of EGF. In a minimal medium supplemented with insulin, transferrin, EGF and fraction III, confluent Ob1771 cells showed a net fibroblastic morphology (Fig.1Ba) similar to that observed in serum-supplemented medium (not shown). The cell morphology observed in fraction I (or II)-supplemented medium was different, whether or not EGF was present : Ob1771 cells appeared flatter and rounder. Cell viability was maintained in fraction IV (or V)-supplemented medium but Ob1771 cells grew poorly and did not reach confluence,

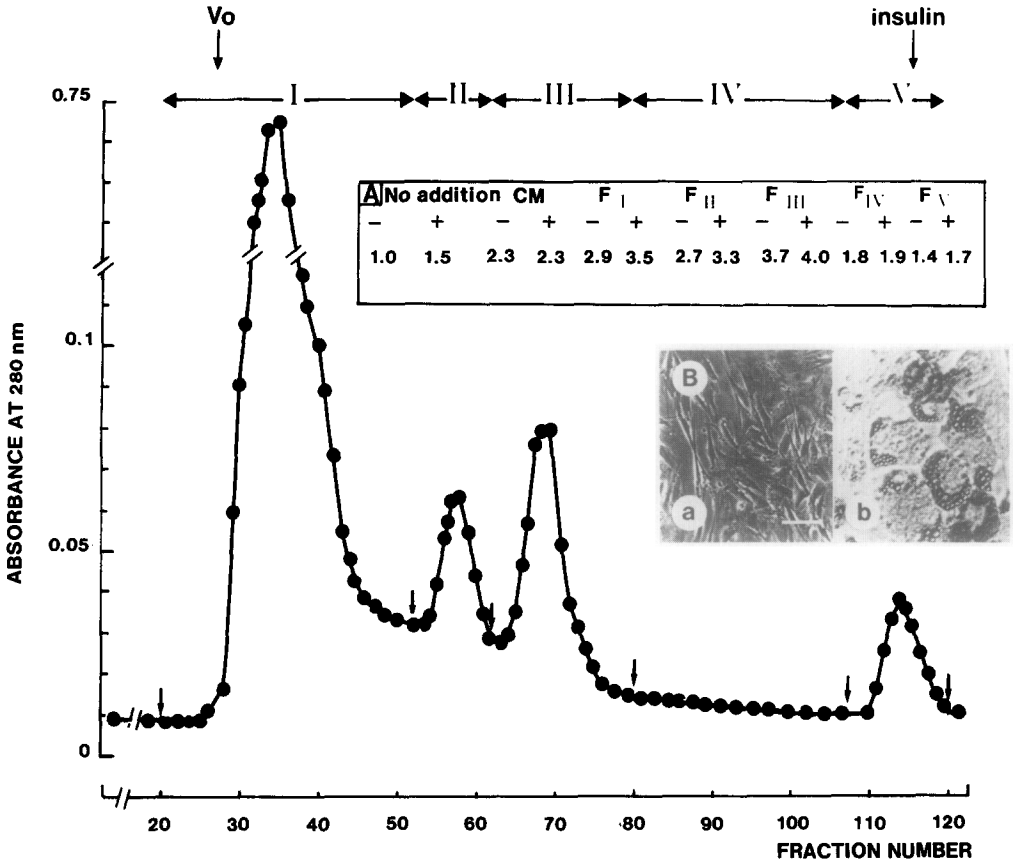

Figure 1 **Elution profile on Bio-Gel P-60 column of fractions from conditioned medium from Ob17MT18 cells**

The conditions used for chromatography are described in the "Materials and Methods" section. Insert **A**, fold increase in the cell number by taking 1.0 the value obtained with no addition ; 5 µl of conditioned medium (**CM**) and of each fraction were added in the absence (-) or presence (+) of 1.7 nM EGF ; Insert **B**, micrographs of Ob1771 cells grown to confluence in the presence of fraction III (**a**) or in the presence of conditioned medium and subsequently shifted following confluence in the presence of fraction V for 16 days (**b**). Identical pictures to **b** were obtained by growing and maintaining the cells after seeding in the presence of conditioned medium for 21 days . Bar equals 20 µm.

whereas EGF showed no effect. Polyacrylamide gel electrophoresis of conditioned medium of both Ob17MT18 cells and fractions I to V performed under reducing conditions, is shown in Figure 2. As expected, the pattern obtained for the conditioned medium, after silver nitrate staining, indicates a rather large heterogeneity. Fraction III appears to be less heterogeneous than fractions I and II and was actually close to homogeneity with a major band of 12-14 K_d (28 K_d under non-reducing conditions, not shown). In contrast to fraction III, the pattern obtained with fraction V indicates the presence of polypeptides, among which should be insulin originally included in the

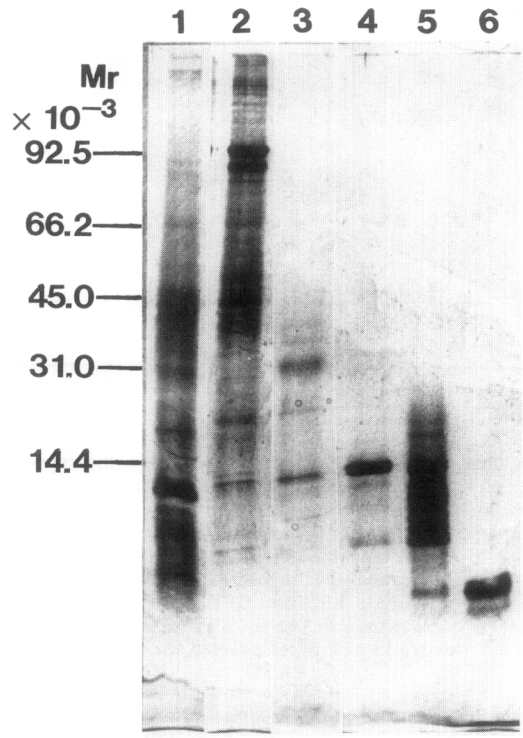

Figure 2 **Silver-stained sodium dodecylsulfate polyacrylamide gels of fractions obtained through purification of the conditioned medium from Ob17MT18 cells**

Samples in lanes 1-6 were treated with 2-mercaptoethanol prior to electrophoresis. Lane 1, 4 μg protein of conditioned medium ; Lane 2, 4 μg protein of fraction I ; Lane 3, 1 μg protein of fraction II ; Lane 4, 0.5 μg protein of fraction III ; Lane 5, 2 μg protein of fraction IV ; Lane 6, 2 μg protein of fraction V. Molecular weight standards used were : phosphorylase b, 92,500 ; bovine serum albumin, 66,200 ; ovalbumin, 45,000 ; carbonic anhydrase, 31,000 ; soybean trypsin inhibitor, 21,500 ; lysozyme, 14,400.

serum free-medium, with apparent molecular weights <10 K_d. The adipogenic activity of the conditioned medium from Ob17MT18 cells is clearly illustrated in Figure 1Bb. After growth in CM-supplemented medium, Ob1771 cells are able to undergo terminal differentiation in the same medium providing T_3, GH and IBMX are present. In the absence of both hormones, lipoprotein lipase, an early marker of adipose conversion, is indeed expressed at very significant activity levels (Fig.3). This observation is in full agreement with results obtained for the differentiation of Ob17 and Ob1771 cells in serum-supplemented medium showing the emergence of LPL activity in T_3- and GH-deprived medium (5). In contrast to LPL the expression of GPDH activity, a marker of terminal differentiation which correlates with triacylglycerol accumulation (3), is fully dependent upon GH and largely dependent upon T_3 addition. The half-maximally and

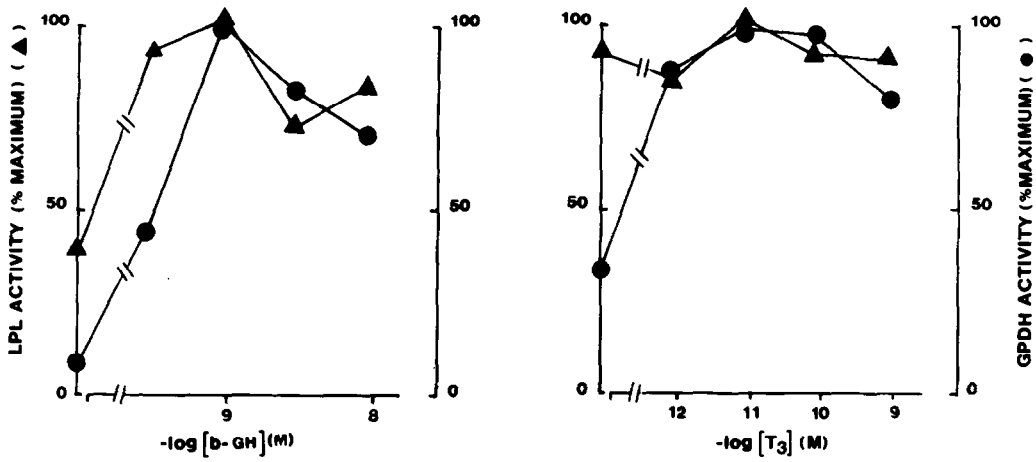

Figure 3 **Dose-response relationships of growth hormone and triiodothyronine to the activities of enzyme markers of adipose conversion**
After growth in serum-free medium supplemented with conditioned medium from Ob17MT18 cells, as described in the "Materials and Methods" section, two series of confluent Ob1771 cells were maintained in the same medium supplemented with 0.15 nM T_3 and the indicated concentrations of b-GH (A) or with 1.4 nM b-GH and the indicated concentrations of T_3 (B). LPL and GPDH activities were determined 16 days after confluence. 100% corresponds in (A) to 6.3 and 330 nmol/min per mg of protein and in (B) to 5.8 and 600 nmol/min per mg of protein for LPL and GPDH, respectively.

maximally effective concentrations of GH (Fig.3A) are in agreement with the binding parameters for GH determined on 3T3-F442A cells and adipocytes (18-20) whereas the maximally effective concentration of T_3 is in agreement with the binding parameters for T_3 determined on Ob17 cells (21). The results of Figure 3 are also in agreement with previous results obtained in serum-supplemented medium (5). The five fractions described above were assayed for adipogenic activity in serum-free medium supplemented with optimal concentrations of GH and T_3. The micrographs of Figure 1Bb and the results of Table II indicate that, after growth in CM-supplemented medium, the activity required for terminal differentiation is mainly concentrated in fraction V (5- to 10-fold purification) and clearly absent from fraction III. Similar results were obtained through fractionation of conditioned medium of MDCK cells (not shown). In contrast to the GPDH activity, the LDH activity, not directly related to lipogenesis and triacylglycerol synthesis remains independent of the added fraction. Additional experiments showed that the adipogenic activity of fraction V was likely involved in the terminal stage of adipose conversion since Ob1771 cells grown for 3 days and then maintained for 8 days in fraction III-supplemented medium possess LPL and GPDH activities of 6.45 and 53 nmol/min per mg of protein, respectively, compared to LPL and GPDH activities of

Table II **Adipogenic activity of fractions from conditioned medium from Ob17MT18 cells**

FRACTION	GLYCEROL-3-PHOSPHATE DEHYDROGENASE	LACTATE DEHYDROGENASE
Experiment I		
CM - Ob17MT18 (4 µg)	324 (1.0)	5114
F I (50 µg)	537 (0.13)	5161
F II (14 µg)	573 (0.5)	5285
F III (12 µg)	203 (0.21)	4070
F IV (6 µg)	259 (0.53)	4709
F V (2.6 µg)	1102 (5.2)	4190
Experiment II		
CM - Ob17MT18 (4 µg)	171 (1.0)	3512
F I (34 µg)	279 (0.2)	3727
F II (8.7 µg)	364 (1.0)	3122
F III (13.6 µg)	161 (0.3)	3225
F IV (3.4 µg)	711 (4.9)	3607
F V (2 µg)	882 (10.3)	3527

Growth and differentiation of Ob1771 cells were obtained under the conditions described in Table I and "Materials and Methods" section. GPDH and LDH activities were determined 16 days after confluence. The values given in parentheses indicate the purification factor of the adipogenic activity for each fraction, by taking a value of 1.0 for the conditioned medium.

20.7 and 673 nmol/min per mg of protein for cells continuously exposed to CM-supplemented medium, respectively. Thus, in the absence of fraction V but in the presence of fraction III alone, an early marker (LPL) had a high activity level in contrast to a late marker (GPDH). Taken together, the above results show that the adipogenic activity of fraction V and the mitogenic activity of fraction III are clearly different molecular entities, as demonstrated also by the results of SDS-PAGE (Fig.2). The activity present in fraction III was shown to be sensitive to a pepsin treatment (0.5 mg/ml final at pH 4.5) which abolished completely the mitogenic activity but on the contrary remained fully stable to heat treatment (15 min at 95°C).

DISCUSSION

Transformation of Ob17 preadipocyte cells by the transfer of the complete region of polyoma virus (Ob17PY) or a modified genome encoding only the middle-T protein (Ob17MT15 and Ob17MT18 cells) allowed us to obtain conditioned media able to support

growth and full differentiation of the original Ob17 cells. Transformation alone did not seem to be sufficient for such a phenomenon to occur since conditioned medium from FR 3T3 cells transformed with the middle-T-only gene of polyoma virus (MTT4 cells) was without effect. Moreover, a preadipocyte origin did not seem by itself to be sufficient since conditioned medium from mouse 3T3-F442A cells was also uneffective on growth and differentiation of Ob1771 cells. The fact that conditioned medium from MDCK cells showed similar properties to that of transformed Ob17 cells is of interest. It might be related to cell transformation and/or to the epithelial origin of this clonal line. The ability of conditioned medium from transformed Ob17 cells to support growth and differentiation was clearly not "clonal line-specific" since such medium was active both on Ob1771 and 3T3-F442A cells (Table I). The results of Bio-Gel P-60 chromatography and SDS-PAGE (Figs.1 and 2), coupled with the results of Tables I and II, strongly support the conclusion that the mitogenic activity is distinct from the adipogenic activity. Preliminary characterization of fraction III has indicated a molecular weight of 27,646 estimated by aminoacid analysis. Fraction III showed a very low TGF β-like activity when tested on NRK cells (1/50 000 of the activity of human TGF-β) and did not show any proteolytic activity when tested against chromophoric substrates. In addition no PDGF-like activity has been detected in growth assays using 3T3 cells sensitive to 0.1 ng PDGF per assay. Since a molecular weight of 12-14 K_d was obtained for the major band present in fraction III after reduction, the results suggest that the protein of 28 K_d is a dimer. Fetuin has been shown previously to behave as a growth-promoting agent of Ob17 cells (2), and it is possible that the 28 K_d protein and fetuin play a similar role. Like the active component(s) purified from serum, the adipogenic activity present in fraction V is of low-molecular weight and active on terminal differentiation. Insulin was excluded as a possible candidate since its addition (up to 4 μM) to the conditioned medium of Ob17MT18 cells or the fraction III-supplemented medium had no effect. A possible relationship between fraction V and TGF-α was also excluded since no competition between fraction V and ^{125}I-human TGF-α could be observed for the binding to NRK cells. In conclusion, the relationships between the active component(s) purified from the conditioned medium of Ob17MT cells and that purified from serum, if any, remain to be investigated.

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