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Potent stimulation of vascular endothelial cell growth by differentiated 3T3 adipocytes

(vascularization/development/conditioned medium/fat cells/serum-free growth)

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ABSTRACT **3T3 cells that have undergone adipose differentiation *in vitro* secrete into the culture medium a potent growth stimulatory activity for bovine aortic endothelial cells. When medium containing 2% fetal calf serum, which does not support significant endothelial cell growth, is conditioned by 3T3-F442A adipocytes, the endothelial cells grow rapidly (doubling time, 24 hr) at a rate equal to the growth rate in 20% fetal calf serum. The potency of the conditioned medium is further shown by the fact that it can be diluted 1:5 with little apparent loss of activity and shows a half-maximal stimulation at 10 μl/ml. Serum is not required for either the secretion of this mitogen by the adipocytes or its action on the endothelial cells, as shown by the fact that the latter are stimulated to divide in serum-free medium conditioned by the adipocytes. The growth stimulatory activity appears to be specific for vascular endothelial cells in that no other cell type examined, including vascular smooth muscle cells and pericytes, are significantly stimulated by medium conditioned by 3T3-F442A cells. Similarly, medium conditioned by no other cell type examined has more than 10% of the activity of medium conditioned by the adipocytes. The specificity and potency of the adipocyte-derived factor suggest that it may play a role in the vascularization of this tissue during development. Preliminary biochemical analysis indicates that the adipocyte factor is nondialyzable and is not inactivated by heat or proteases. The protease insensitivity distinguishes the adipocyte growth stimulatory activity from the low levels of activity secreted by fibroblasts and preadipocytes, suggesting that the adipocyte mitogen is a product specifically related to the differentiation process.**

Vascularization of tissues occurs during many important and diverse biological processes including normal embryonic development, wound healing, inflammation, and neoplasia. Because vascularization involves activation of endothelial cell motility and growth (1, 2) much attention has been focused on factors that stimulate these processes *in vitro*. Recently, Folkman *et al.* (3) and Zetter (4) have shown that medium conditioned by tumor cell cultures stimulates the growth and motility of cultured capillary endothelial cells. Harris *et al.* (5) have reported that a protein produced by cultured bovine aortic endothelial cells (BAEC) stimulates the growth of other BAEC and also stimulates vascular smooth muscle cells. Birdwell *et al.* (6) found that BAEC growth was enhanced by BALB/c 3T3 feeder layers or conditioned medium. The molecular basis and biological significance of these mitogenic and motility stimulating activities require further investigation.

We are interested in vascularization during normal tissue development. As a model we have used BAEC and differentiating 3T3-F442A adipocytes (7). We chose this system for several reasons: (*i*) adipose tissue is very highly vascularized, comparable to or greater than the extent of vascularization of striated muscle (8), (*ii*) 3T3-F442A cells undergo conversion from fibroblast-like preadipocytes to adipocytes which have most of the characteristics of fat cells in adipose tissue (9), and (*iii*) undifferentiated 3T3-F442A, when injected into athymic *nude* mice, form a highly vascularized fat pad at the site of injection (10), suggesting that these cells possess all of the properties required to stimulate vascularization.

We report here that differentiated 3T3 adipocytes produce a potent mitogen which is specific for vascular endothelial cells. This mitogen is not sensitive to proteases and thus can be distinguished from the lower levels of mitogenic activity produced by fibroblasts and preadipocytes.

MATERIALS AND METHODS

All chemicals and enzymes were obtained from Sigma unless otherwise noted. Chinese hamster embryo fibroblast (CHEF 18-1) cells were obtained from Ruth Sager (Sidney Farber Cancer Institute). 3T3-F442A, 3T3-C2 (7), 3T3-M (uncloned 3T3), and XB cells (11) were obtained from Howard Green (Massachusetts Institute of Technology). These cells, along with baby hamster kidney-21/C13 (BHK), NIH-3T3, BALB/c 3T3, and bovine aortic smooth muscle cells were grown in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% calf serum at 37°C in a 10% CO_2/90% air atmosphere. To minimize adipose conversion in 3T3-F442A stocks, cells were grown in DME medium supplemented with 9% cat serum and 1% calf serum (12). BAEC, bovine aortic smooth muscle cells, and rat aortic smooth muscle cells were isolated and characterized as described (13–17). Rat aortic smooth muscle cells were grown in RPMI-1640 medium containing 20% fetal calf serum at 37°C in a 10% CO_2/90% air atmosphere. BAEC were grown either in DME medium containing 10% calf serum or RPMI-1640 supplemented with 20% fetal calf serum at 37°C in a 10% CO_2/90% air atmosphere.

Conditioned medium was collected from differentiated 3T3-F442A cells which were grown and maintained for 6–7 days after confluence in medium containing 10% fetal calf serum, at which time adipose conversion had reached a maximum. Cells were washed three times in DME medium, and 10 ml of DME medium with or without a small amount of serum was placed on each 100-mm dish. After 24 hr the conditioned medium was removed and filtered through a 0.22-μm filter. This filtration had no effect on BAEC stimulatory activity. The conditioned medium was stored at 4°C in the dark for up to 2 months with little or no loss of activity.

To assay the mitogenic activity in conditioned medium from differentiated 3T3-F442A or other cells, 8×10^3 BAEC between passages 3 and 10 were plated into 16-mm multiwell plates (Costar). After 24 hr, control cultures were put in medium containing a concentration of serum that supported less than

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Abbreviations: BAEC, bovine aortic endothelial cells; DME medium, Dulbecco's modified Eagle's medium.

one doubling of BAEC in the 5 days the mitogenesis assay was carried out. This serum concentration was determined for each BAEC isolation and ranged from 0.4% to 2% fetal calf serum (for BAEC grown in RPMI containing 20% fetal calf serum) and from 0.1% to 0.4% calf serum (for BAEC grown in DME medium containing 10% calf serum). Experimental cultures were placed in low-serum medium containing different concentrations of conditioned medium. All cultures were fed after 2 days. Cell number was measured at 24-hr intervals for 5 days by using a Coulter Counter.

RESULTS

Stimulation of BAEC Growth by Conditioned Medium from Differentiated 3T3-F442A. When BAEC growing exponentially in medium containing 20% fetal calf serum were placed in medium containing 1% fetal calf serum, their growth was greatly reduced within 1 day and almost completely arrested within 2 days after the shift to low serum (Fig. 1). In contrast, when serum-free medium conditioned for 24 hr by differentiated 3T3-F442A cells was mixed 1:1 with fresh medium containing 2% fetal calf serum and applied to the cells, they proliferated rapidly, with approximately the same doubling time as in 20% fetal calf serum (24 hr). For each isolation of endothelial cells, the serum concentrations producing cessation of growth (0.1–2%) and optimal growth (10% calf serum or 20% fetal calf serum) were determined. In more than 20 separate experiments involving different batches of cells and conditioned medium, a marked stimulation of BAEC growth by adipocyte-conditioned medium was always observed. Quantitatively, the stimulation above the low serum background, which supported little or no growth, varied from a doubling time 30% longer to one 25% shorter than that of BAEC in the optimal normal medium for that batch of cells. In all experiments, BAEC in low-serum conditioned medium went through three or more doublings while BAEC in unconditioned low-serum medium usually went through less than one doubling.

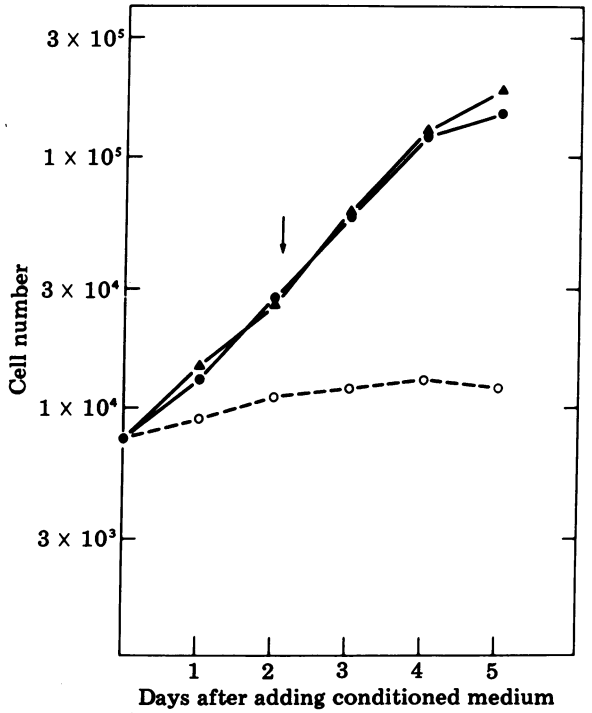

FIG. 1. Effect of conditioned medium from differentiated 3T3-F442A on BAEC growth. BAEC were plated and the mitogenic effect of differentiated 3T3-F442A-CM was assayed. Conditioned medium (serum-free) was mixed 1:1 with RPMI containing 2% fetal calf serum. Cells were re-fed at day 2 (arrow). ●—●, RPMI containing 20% fetal calf serum; o---o, RPMI containing 1% fetal calf serum; ▲—▲, differentiated 3T3-F442A-conditioned medium mixed 1:1 with RPMI containing 2% fetal calf serum.

Target Cell Specificity. Medium conditioned by differentiated 3T3-F442A cells was tested for its ability to stimulate the growth of other cell types, including primary and established fibroblasts and nonfibroblastic cell types (Table 1). Calf aortic smooth muscle cells and BHK were not stimulated at all by the conditioned medium. Several other types, including rat aortic smooth muscle, NIH Swiss 3T3, BALB/c 3T3, and rat capillary pericytes, were stimulated to a small extent. No cell type examined showed more than 20% of the stimulation shown by BAEC at a concentration of conditioned medium of 500 μl/ml, or 5% of that of BAEC at 200 μl/ml. In addition to these cell types, we have examined the effect of adipocyte-conditioned medium on XB cells. The growth of these mouse teratomal keratinocytes is known to require a factor present in medium conditioned by fibroblasts (11). We have found that conditioned medium from the 3T3-F442A adipocytes does not support the growth of these cells significantly better than does medium conditioned by 3T3-C2 cells, a clone which undergoes adipose differentiation with very low frequency (7). The absence of significant stimulatory activity in the medium conditioned by differentiated 3T3-F442A toward other cell types strongly suggests that this mitogenic activity is specific for the vascular endothelial cells. However, we cannot rule out the possibility that other cell types have the potential to respond to the adipocyte factor, but they require other factors not present in

Table 1. Effect of conditioned medium from differentiated 3T3-F442A on growth of different cell types

Cell type	Concentration of CM in low-serum medium, μl/ml*	% of growth in normal medium
BAEC	500	105
	200	96
	10	52
Rat aorta smooth muscle	500	10
	200	0
Calf aorta smooth muscle	500	0
	200	0
BHK	500	0
	200	0
NIH Swiss 3T3	500	10
	200	0
BALB/c 3T3	500	20
	200	5
Rat capillary pericytes	500	10
	200	0

Cells were plated in their normal growth medium at 6×10^3 per 16-mm well in 24-well tissue culture dishes. At 24 hr later, the medium was replaced with medium conditioned by differentiated cells, containing 0.4% serum. Growth in 0.4% serum alone was used as the zero baseline. Growth in normal medium (10% calf serum or 20% fetal calf serum) for each cell type was taken as 100%. Data were taken at day 3 after addition of conditioned medium, when cells were in exponential growth.

* CM, conditioned medium.

sufficient quantities in the low serum concentration used in the assay system.

Producer Cell Specificity. Medium conditioned by other cell types has been reported to stimulate BAEC growth (5, 6). We compared the effect of conditioned medium from differentiated 3T3-F442A with conditioned medium from various other cell types on the growth of BAEC (Table 2). All media contained 0.4% calf serum during the 24-hr conditioning period. Conditioned medium from undifferentiated 3T3-F442A grown and maintained in cat serum (12), 3T3-C2, BALB/c 3T3, exponential BHK, and exponential BAEC cells stimulated BAEC growth at a concentration of 500 μl/ml. At lower concentrations of conditioned medium the stimulation was substantially reduced. Conditioned medium from differentiated 3T3-F442A at 500 μl/ml produced stronger stimulations than did any other conditioned medium tested. In addition, it could be diluted to at least 200 μl/ml before mitogenic activity began to decrease substantially. Comparison of the concentrations of conditioned medium that produce half maximal stimulation shows that medium conditioned by differentiated 3T3-F442A is at least 10 times more potent than any other conditioned medium tested. This is true when expressed on the basis of the number of cells or on the amount of cell protein conditioning the medium. Similar results were obtained when differentiation of 3T3-F442A was prevented by maintaining them in a growing state. Stimulations of BAEC growth similar in magnitude and potency to 3T3-F442A adipocytes have also been observed with medium conditioned by differentiated 3T3-L1 cells, another adipogenic 3T3 clone.

Table 2. Effect of conditioned medium from different cell types on BAEC growth

Cell type	Concentration of CM in low-serum medium, μl/ml*	% of growth in normal medium	Concentration at half-maximal stimulation, μl/ml
Differentiated	500	105	
3T3-F442A	200	96	10
	10	52	
Undifferentiated	500	90	
3T3-F442A	200	64	100
	10	10	
3T3-C2 in fetal calf serum	500	92	
	200	55	180
	10	7	
3T3-C2 in cat serum	500	88	
	200	49	210
	10	6	
Swiss 3T3	500	10	
	200	0	
BALB/c 3T3	500	51	500
	200	18	
BAEC	500	25	
	200	5	
Exponential BAEC	500	80	
	200	37	400
	10	0	
BHK	500	0	
	200	0	
Exponential BHK	500	49	500
	200	14	
Chinese hamster	500	0	
embryo fibroblasts	200	0	
Rat aorta smooth	500	12	
muscle	200	0	

Conditioned medium containing 0.4% calf serum was collected from different cell types in confluent culture except where noted. Undifferentiated 3T3-F442A was grown and maintained in medium containing 9% cat serum and 1% calf serum. The amount of medium conditioned by each cell type was adjusted to keep the ratio of cell number to volume of medium constant. Growth in 0.4% serum was used as the zero baseline. Growth in normal medium is taken as 100%. Data are taken from day 3 after addition of conditioned medium, when cells were in exponential growth.
* CM, conditioned medium.

It is possible that other cell types may produce this factor but they require higher serum concentrations than used here. Although we cannot rule out this possibility completely, we tested the ability of BAEC, rat aortic smooth muscle cells, 3T3-C2, and undifferentiated 3T3-F442A to produce an endothelial cell stimulatory activity in medium containing 2% serum. None of these cell types produced more stimulatory activity under these conditions than they did in 0.4% serum.

The mitogenic activity produced by undifferentiated 3T3-F442A and 3T3-C2 could be a result of their fibroblastic nature (see BALB/c 3T3) or it could be due to a small amount of adipose conversion under these conditions. However, when 3T3-C2 was grown and maintained in medium containing fetal calf serum, which favors adipose conversion, it produced approximately the same amount of activity as it did when maintained in cat serum (Table 2). It is interesting to note that essentially no mitogenic activity was found in conditioned medium from Swiss-3T3 cells, the parent line of the F442A and C2 clones. This confirms similar observations by Birdwell *et al.* (6).

Stimulation of BAEC Growth in Serum-Free Medium. The 3T3-F442A adipocytes did not require serum to secrete the stimulatory activity (not shown). To determine if the presence of serum was required for BAEC to respond to this factor, the

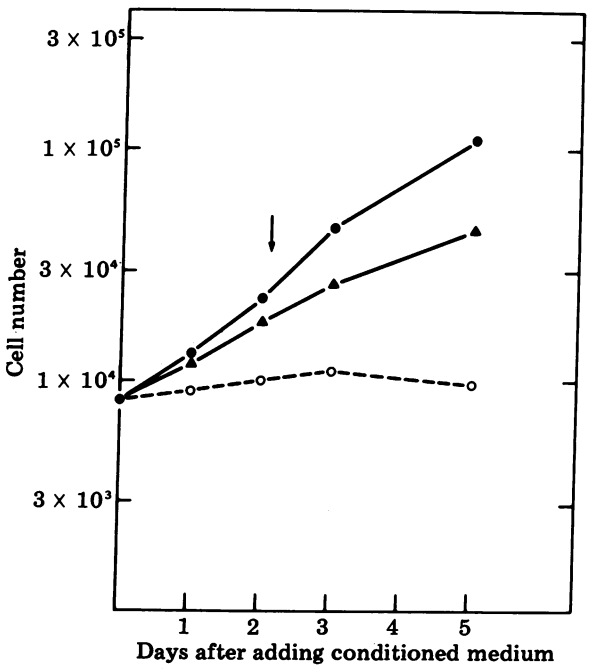

FIG. 2. Stimulation of BAEC growth in serum-free conditioned medium. Differentiated 3T3-F442A were washed three times with DME medium. Serum-free conditioned medium from differentiated 3T3-F442A was mixed 1:1 with fresh DME medium. This mixture was added to BAEC which had been washed three times with DME medium. Cell number was measured by using a Coulter Counter. Cells were re-fed at day 2 (arrow). ●—●, DME medium containing 10% calf serum; O---O, serum-free DME medium; ▲—▲, serum-free conditioned medium from differentiated 3T3-F442A mixed 1:1 with fresh DME medium.

cells were washed and incubated in serum-free conditioned medium which was mixed 1:1 with fresh serum-free medium. This mixture supported BAEC growth (Fig. 2). Even after refeeding with the serum-free mixture, some growth occurred. Although we cannot rule out contributions from trace amounts of residual serum, no added serum was required for the mitogenic activity to be secreted or expressed. By the second day in serum-free conditioned medium, the BAEC began to elongate and vacuolate and after 4 days the cells looked quite unhealthy. Although serum is not required for the mitogenic factor to act, it does appear to be required for prolonged survival of the cells.

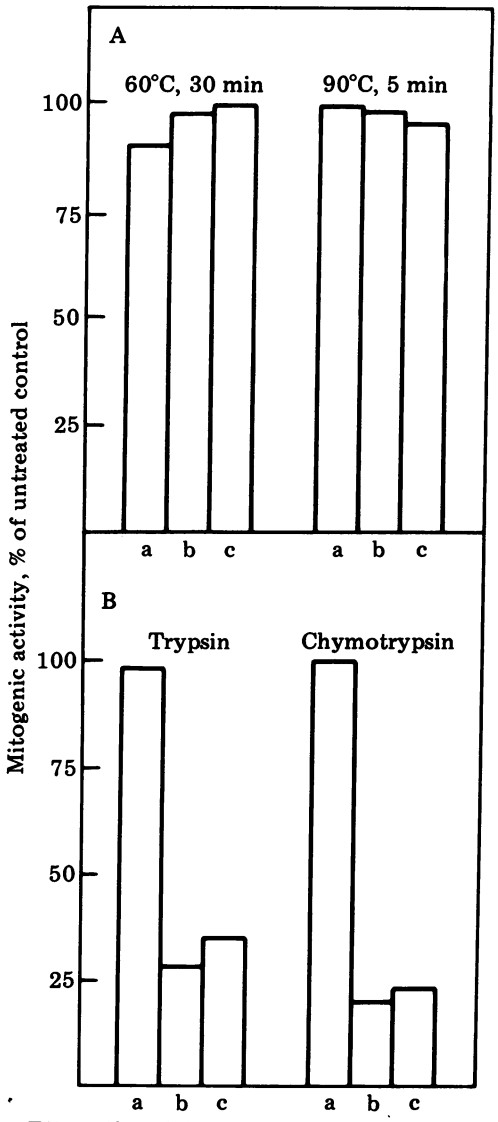

FIG. 3. Effect of heat (*A*) and protease (*B*) treatments on mitogenic activity in conditioned medium. Values for untreated conditioned media were set at 100%. Data shown here are based on cell number at day 3 after addition of conditioned medium in the range of linear relationship between cell number and conditioned medium concentration (200 μl/ml for undifferentiated F442A and C2, 50 μl/ml for differentiated F442A). Experiments using other dilutions of conditioned medium yielded similar results. Trypsin and chymotrypsin treatment was carried out at 15 μg/ml. Digestion was for 60 min at 37°C and was terminated by addition of a 10-fold weight excess of soybean trypsin inhibitor (for trypsin) or chicken eggwhite ovoinhibitor (for chymotrypsin). Controls showed no effect of preincubated inhibitor–protease complex on cell growth, indicating that the effect of the proteases was on the conditioned medium and not on the BAEC. Conditioned medium were from: a, differentiated 3T3-F442A; b, undifferentiated 3T3-F442A; c, 3T3-C2.

Preliminary Biochemical Characterization of the Mitogenic Activity. The mitogenic activity in conditioned medium from differentiated 3T3-F442A is nondialyzable and nonfilterable (Millipore filtration membrane with a nominal cut-off of molecular weight 10,000). When conditioned medium from differentiated 3T3-F442A was heated at 60°C for 30 min or at 90°C for 5 min, no activity was lost (Fig. 3A, a). The same was true for conditioned medium from undifferentiated 3T3-F442A and 3T3-C2 (Fig. 3A, b and c).

Medium conditioned by differentiated 3T3-F442A and treated with either trypsin or chymotrypsin (15 μg/ml, 60 min, 37°C) retained virtually all its BAEC stimulatory activity (Fig. 3*B*, a). In contrast to this, conditioned medium from undifferentiated 3T3-F442A or 3T3-C2 cells lost 65–80% of its mitogenic activity (Fig. 3*B*, b and c). Experiments carried out with a higher concentration of chymotrypsin (40 μg/ml) gave no additional loss of activity in any sample, indicating that a digestion end point was reached. All assays were done at several concentrations in the linear range of activity. Thus, most of the small amount of mitogenic activity in conditioned medium from undifferentiated 3T3-F442A and 3T3-C2 cells appears different from the mitogenic activity secreted by differentiated 3T3-F442A in its sensitivity to proteases. It is possible that the 3T3-F442A adipocyte factor is also protease sensitive but that a protease inhibitor is present in the conditioned medium. We have investigated this possibility by examining the hydrolysis of the protease substrate benzoyltyrosine ethyl ester by chymotrypsin in conditioned medium and find no detectable loss of protease activity. This suggests that the BAEC stimulatory factor produced by 3T3-F442A adipocytes is distinct from the protease-sensitive activity produced by the 3T3-C2 and undifferentiated 3T3-F442A cells. The specific molecular nature of the adipocyte-derived activity remains to be elucidated.

DISCUSSION

This paper reports that differentiated 3T3 adipocytes produce a factor (or factors) which strongly stimulates the growth of BAEC. This stimulation is highly specific in that it does not significantly stimulate any other cell type examined (Table 1). Similarly, conditioned medium from no other cell type examined here (Table 2) stimulated BAEC with more than 10% of the activity of that from the adipocytes. The specificity of the adipocyte-conditioned medium–BAEC interaction and its potency (half-maximal at 1:100 dilution) strongly suggests that this activity may play a role in the vascularization process of developing adipose tissue *in vivo*. The large increase in amount of activity during adipose conversion *in vitro* and the fact that the adipocyte activity is distinguishable from most of the preadipocyte activity by the former's insensitivity to proteases indicates that the production of the adipocyte factor is closely linked to the cell differentiation process. This is consistent with the observation that adipocyte differentiation *in vivo* is tightly coordinated with extensive vascularization of the developing fat pad (18).

The migration and growth of endothelial cells are two important and distinct processes during tissue vascularization (1, 2). In the present study, our attention has been focused solely on the growth of the vascular endothelial cells. It will be important to examine whether the adipocyte-conditioned medium stimulates migration of vascular endothelial cells and, if so, whether the mitogenic activity is distinct from motility-stimulating activity. Similarly, since vascularization of adipose tissue occurs via invasion of small vessels, the effect of the adipocyte-conditioned medium on endothelial cells from small vessels and capillaries is of obvious importance. In preliminary experiments, we have found that the adipocyte-conditioned

medium strongly stimulates the growth of bovine adrenal capillary cells (kindly supplied by B. Zetter). This stimulation is half-maximal at a concentration similar to that required for half-maximal stimulation of the aortic endothelial cells (10 μl/ml). Thus, the effect of the adipocyte-derived mitogen may be general for vascular endothelial cells.

A number of investigators have reported factors that stimulate endothelial cell growth *in vitro* or vascularization *in vivo*. The sources for these factors include tumors and cultured tumor cells (2, 19, 20), synovial fluid (21), retina (22), cultured endothelial cells (5), BALB/c 3T3 cells (6), corpus luteum (23), and macrophages (24). However, the important question of whether these mitogenic factors are specific for vascular endothelial cells has not been thoroughly explored in most cases. Comparison of the biochemical properties of the adipocyte-produced mitogen and other growth factors specific for vascular endothelial cells may provide insights into the relationship between different types of vascularization.

Finally, it may be possible to use 3T3-F442A adipocyte-conditioned medium as a tool for culturing endothelial cells from different tissue sources because of its potency and specificity. A similar approach (using tumor conditioned medium) has been used successfully by Folkman *et al.* (3) to isolate and grow capillary endothelial cells.

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