Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 194-202

EGF INHIBITS THE DIFFERENTIATION OF ADIPOCYTE PRECURSORS IN PRIMARY CULTURES

Ginette Serrero

W. Alton Jones Cell Science Center, Inc.
10 Old Barn Road,
Lake Placid, NY 12946

Received April 24, 1987

SUMMARY: Adipocyte precursors, isolated from inguinal fat pads of 2-day old NBR rats, proliferate and differentiate in defined medium. EGF stimulates the proliferation of adipocyte precursors with an ED_{50} of 2 ng/ml and inhibits their differentiation with an ED_{50} of 0.45 ng/ml. EGF has no effect on differentiated adipocytes. EGF binding studies indicate that adipocyte precursors have two classes of receptors (Kd 4 x 10^{-11} M, 4800 receptors/cell and Kd 1.4 x 10^{-9} M, 22,000 receptors/cell). TGF-α has the same effect as EGF on adipocyte precursors. In contrast, basic and acidic FGF stimulate adipocyte precursor proliferation without inhibiting their differentiation.

One of the important questions in the study of adipose tissue development is to identify the factors and hormones controlling the proliferation and differentiation of normal adipocyte precursors. The use of established adipogenic cell lines cultivated in serum-containing or in serum-free media has provided a powerful approach to identify some of the hormones and growth factors which modulate adipose differentiation (1-9). Recently, we have demonstrated that an insulin-independent cell line isolated from the adipogenic cell line 1246 (9,10) and which has lost the ability to differentiate, produced in its conditioned medium a factor specifically inhibiting adipose differentiation of the parent cell line (11) and co-eluting with a TGF-α-like activity (Serrero and Yamada, manuscript in preparation). Based on these observations, we examined if TGF-α and its counterpart EGF could act as physiological regulators of adipose differentiation. As we had previously demonstrated that adipocyte precursors freshly isolated from inguinal fat pads in primary culture could proliferate and differentiate in defined medium (12), experiments were performed to determine if EGF and TGF-α would affect the proliferation and differentiation of normal adipocyte precursors.

0006-291X/87 $1.50

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium (DME), Ham's nutrient F12 and gentamycine were purchased from Gibco (Grand Island, New York). Disposable plasticware was used for all cell culture experiments. The following compounds were obtained from Sigma (St. Louis, MO): collagenase, bovine insulin, human transferrin, poly-d-lysine, sodium bicarbonate, dihydroxyacetone phosphate, nicotinamide adenine dinucleotide reduced form (NADH), chloramine T, tyrosine sodium phosphate. N-2-hydroxyethylpiperazine-N'-2-ethonesulfonic acid (HEPES) was obtained from Research Organics (Cleveland, OH). Bovine pituitary fibroblast growth factor (FGF), human plasma fibronectin and mouse submaxillary gland EGF (receptor grade) were purchased from Collaborative Research (Waltham, MA). Pure acidic and basic FGF were a gift from Dr. Wallace McKeehan (W. Alton Jones Cell Science Center, Lake Placid, NY).

METHODS

Cell Culture

Adipocyte precursors were isolated from the stromal vascular fraction of inguinal fat pads of 2 day-old NBR rats as described previously (12). The cells were counted with a hemocytometer and inoculated at a density of 1-2 x 10^4 cells/cm^2 in 35 mm dishes that had been previously coated with polylysine according to the method of McKeehan and Ham (13). The cells were plated in a 1:1 mixture of DME-F12 medium supplemented with human plasma fibronectin (2.5 µg/ml), bovine insulin (10 µg/ml), human transferrin (10 µg/ml) and FGF (10 ng/ml).

Proliferation and differentiation assays

Growth was measured by counting the cell number after trypsinization using a Coulter Counter. Differentiation was followed by measuring the increase of glycerol-3-phosphate dehydrogenase specific activity (G3PDH). For this purpose, the cells were harvested as described previously (12) and measurement of G3PDH was performed according to Wise and Green (14).

[^{125}I]EGF binding to adipocytes precursors

Receptor grade EGF was iodinated by the chloramine T method (15). [^{125}I]EGF binding was performed on monolayers of adipocytes precursors after 4 days in culture, time at which binding has reached a maximum. Cells were washed twice with binding buffer consisting of DME medium pH 7.4 supplemented with 1 mg/ml of bovine serum albumin (BSA). For the Scatchard analysis equilibrium binding was performed at 4°C during 4 hr in 1 ml of binding buffer containing 10^4 cpm of [^{125}I]EGF (256 µCi/µg) for concentrations of EGF below 4 x 10^{-10} M and 10^5 cpm of [^{125}I]EGF for concentrations above 4 x 10^{-10} M. Concentrations of unlabeled EGF were varied from 5 x 10^{-12} M to 2 x 10^{-7} M. At the end of the binding, plates were washed three times with cold binding buffer and once with PBS. Cells were dissolved in 0.1 N NaOH and counted using a γ counter. Cell number from duplicate dishes was determined using a Coulter counter as described above.

RESULTS AND DISCUSSION

Adipocyte precursors, freshly isolated by collagenase treatment from inguinal fat pads of 2-day old NBR rats (12) were inoculated in 4F medium consisting of DME-F12 medium supplemented with fibronectin (2.5 ug/ml),

insulin (10 ug/ml), FGF (10 ng/ml) and transferrin (10 ug/ml). At low cell density, the adipocyte precursors displayed a fibroblastic morphology. After 7 days in culture, 90% of the cells had rounded up, accumulated triglycerides in their cytoplasm and acquired the typical morphology of differentiated adipocytes (12). Glycerol-3-phosphate dehydrogenase (G3PDH) specific activity was followed as a marker of adipose differentiation. G3PDH level increased to 600 mU/mg protein in the differentiated cells after 7 days of culture in the 4F medium (12). The effect of EGF was studied by adding increasing concentrations of EGF to the cells cultivated in 4F medium. After 7 days, the cell number and G3PDH activity were determined and compared to the control represented by cells cultivated in 4F medium only. EGF stimulated the proliferation of the adipocyte precursors when added at a concentration above 0.5 ng/ml. Half maximal stimulation was observed with an EGF concentration of 2 ng/ml, and maximal stimulation with an EGF concentration of 10 ng/ml (Figure 1a). In the presence of EGF, the cells maintained an elongated and spindle-shaped morphology characteristic of the undifferentiated cells and did not accumulate triglycerides. In these conditions, the increase of G3PDH specific activity observed in the cells cultivated in the 4F medium was inhibited by EGF in a dose dependent fashion. Half maximal inhibition occurred at an EGF concentration of 0.45 ng/ml and maximal inhibition at 5 ng/ml (Fig. 1b). As 4F medium in which the cells are cultivated contains FGF previously shown to stimulate the proliferation of the adipocyte precursors (12), the effect of EGF on proliferation and differentiation was reexamined in the absence of FGF. It was shown that EGF dose-response curves for growth and differentiation were similar to the ones obtained for cells cultivated in the presence of FGF (data not shown). The data suggested that the absence of growth stimulatory effects observed at low concentrations of EGF were not being masked by the growth supported by FGF in the 4F medium.

Our results indicated that EGF affected differentiation of adipocyte precursors. We then examined if EGF had any action once differentiation had occurred. As shown in Table 1a, EGF ability to block differentiation was decreased if it was added to the plates after 5 days when the cells had started to undergo differentiation (day 5). Moreover, EGF had no inhibitory effect on the cells once differentiation was completed (Table 1b). These results indicate that EGF effect is primarily on undifferentiated precursor cells and cannot be observed once the cells have started to differentiate.

As a primary culture system was used to investigate EGF effect, another possible explanation of the results obtained was that EGF stimulated the proliferation of a contaminating cell type also originated from the stromal vascular fraction and distinct from the adipocyte precursors. In this case, EGF would not act directly by inhibiting the differentiation of adipocyte

Table 1. Effect of EGF on Undifferentiated Adipocyte Precursors and Differentiated Adipocytes

Conditions	Cell Number x 10^{-3}	G3PDH mU/mg protein
a. 4F	626	601
EGF day 1	988	80
day 5	998	442
b. 4F	ND	620
EGF day 7	ND	604

ND - Not Determined

a. Cells were plated in 4F medium as described in Materials and Methods. 10 ng/ml of EGF was added to the 4F medium at day 1 and at day 5. Cells were collected 7 days after the addition of EGF to measure cell number and G3PDH specific activity.

b. 10 ng/ml of EGF was added at day 7 to cells cultivated in 4F medium and which were fully differentiated. EGF was maintained in the 4F medium for 7 additional days until the cells were harvested to measure G3PDH specific activity.

precursors but rather by allowing a different cell type to proliferate. This possibility was investigated by two types of experiments. In the first experiment, we examined whether the cells exposed to EGF during the primary cultures would maintain their ability to differentiate in secondary cultures when they were replated at low cell density in 4F medium only. If this was the case, it would indicate that EGF effect was reversible and that the cells which had been stimulated to grow in the presence of EGF were in fact the adipocyte precursors. In the second experiment, we determined if EGF could inhibit the differentiation in secondary cultures of cells that had been cultivated in 4F medium only during the primary cultures. As shown previously, these culture conditions (4F medium) are quite specific for supporting the proliferation and differentiation of adipocyte precursors in primary cultures since 90-100% of the cells plated in the 4F medium differentiate into adipocytes after 7 days in culture. Thus, if EGF inhibited differentiation in these conditions, this would indicate that it acted directly on the adipocyte precursors. The results in Table 2a demonstrate that more than cells exposed for 5 days to EGF during the primary culture can undergo differentiation when cultivated in the 4F medium in the secondary cultures. Table 2b shows that EGF inhibits by 90% the differentiation of adipocyte precursors that have been cultivated for 4 days in 4F medium prior to be passaged. These data indicate that the effect of EGF on adipose differentiation is reversible and that EGF acts directly by blocking the differentiation of the adipocyte precursors. Based on these data, we investigated whether the inhibition of differentiation was due to the growth stimulation of adipocyte precursors by EGF. When the effect of EGF on both processes are compared (Figure 1a and 1b), it is quite apparent that the dose response curves are different. EGF inhibits adipocyte precursor differentiation by 50% at concentrations that do not stimulate their

Table 2. Differentiation ability of secondary cultures

Conditions	G3PDH (mU/mg protein)
a. 4F	286
4F + EGF	27
b. 4F	330
4F + EGF	30

a. During the primary culture, the cells were cultivated in 4F medium in the presence of EGF (10 ng/ml). After 4 days the cells were washed, detached with phosphate buffer saline pH 7.4 containing 1 mM EDTA. Cell suspension was washed twice with DME-F12 medium by centrifugation. The cells were plated at a density of 10^4 cells/cm^2 either in 4F medium alone or containing 10 ng/ml of EGF. 7 days later, the cells were harvested and G3PDH specific activity determined.
b. Same as above except that during the primary culture, the cells were cultivated in 4F medium.

proliferation. These data would suggest that the action of EGF on differentiation must not be through its effect on growth. To further investigate this possibility, we reexamined the effect of FGF on adipose differentiation. Athough commercially available FGF is one of the components of the 4F medium, the effect of pure basic pituitary FGF and acidic brain FGF (kindly provided by Dr. Wallace McKeehan) was investigated. At concentrations up to 1 nM, basic FGF stimulated by 2-fold the growth of adipocyte precursor cells,

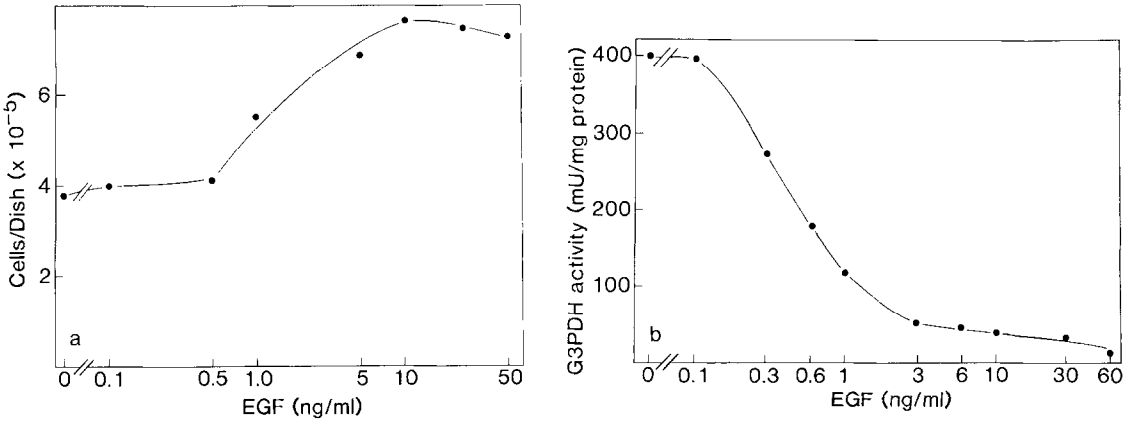

Fig. 1. Effect of EGF on the proliferation and differentiation of adipocyte precursors.

a. Proliferation assay: Adipocyte precursors were cultivated in the 4F medium as described in Materials and Methods. EGF was added on day 1 and maintained throughout the whole experiment. Medium was changed on day 1 and day 4. Cells were collected by trypsinization on day 7 and counted.

b. Differentiation assay: The culture conditions were the same as above. At day 7, the cells were harvested and glycerol-3-phosphate dehydrogenase activity (G3PDH) measured as described previously (12).

Table 3. Effect of FGF and TGF-α on adipocyte precursor proliferation and differentiation

Conditions		Cells/dish	G3PDH
3F		100	100
EGF	0.1 nM	110	34
	1 nM	160	12
bFGF	0.1 nM	150	215
	1 nM	220	173
aFGF	0.1 nM	120	105
	1 nM	130	110
TGF-α	0.1 nM	109	40
	1 nM	120	22

Adipocyte precursors were cultivated in defined medium except that FGF was omitted (3F). Receptor grade EGF, basic pituitary FGF, (bFGF), acidic brain FGF, (aFGF) and synthetic rat TGF-α, were added to the cultures on day 1 and day 4. Cells were collected on day 7 in order to measure cell number and G3PDH specific activity. The values are expressed relatively to the controls (cells maintained in 3F medium, in the absence of FGF). Cell number measured in the 3F plates represented by 100 was 4 x 10^5 cells. G3PDH specific activity was 375 mU/mg protein.

whereas acidic FGF had a lesser effect (Table 3). Interestingly, under these culture conditions where cell proliferation was maximally stimulated, the adipose differentiation was also stimulated. In contrast, EGF in the same conditions inhibited G3PDH specific activity by 86%. This result strongly indicates that the inhibition of adipose differentiation by EGF is not simply due to growth stimulation of adipose precursor cells, but presumably through a different mechanism. These data of the FGF effect on rat adipocyte precursors are in agreement with the findings of Broad and Ham using sheep preadipocytes in primary cultures (16).

As EGF inhibited the differentiation of adipocyte precursors in primary culture, we examined the effect of transforming growth factor TGF-α. TGF-α is a polypeptide of molecular weight 7.5 kDa purified from retrovirus-transformed cells and tumor cells (17). It is structurally and functionally related to EGF and competes with EGF for binding to EGF receptor (18-19). As shown in Table 3, synthetic rat TGF-α was able to block differentiation of adipocyte precursors in a dose-dependent fashion. Maximal inhibition was obtained with a TGF-α concentration of 1 nM, similar to EGF. Moreover, the fact that synthetic TGF-α was able to block adipose differentiation similarly to EGF indicated that the effect of EGF was not due to a contaminating impurity that could be present in the commercially purified EGF samples.

Binding of [^{125}I]labeled EGF was performed on adipocyte precursors cultivated in 4F medium. Time-course experiment in Figure 2a showed that EGF binding increased during the proliferation phase and reached an optimal value 4 days after plating when the cells were still undifferentiated. Thereafter, the specific binding decreased when the cells started to differentiate. This decrease in EGF binding could account for the decreased effect of EGF on

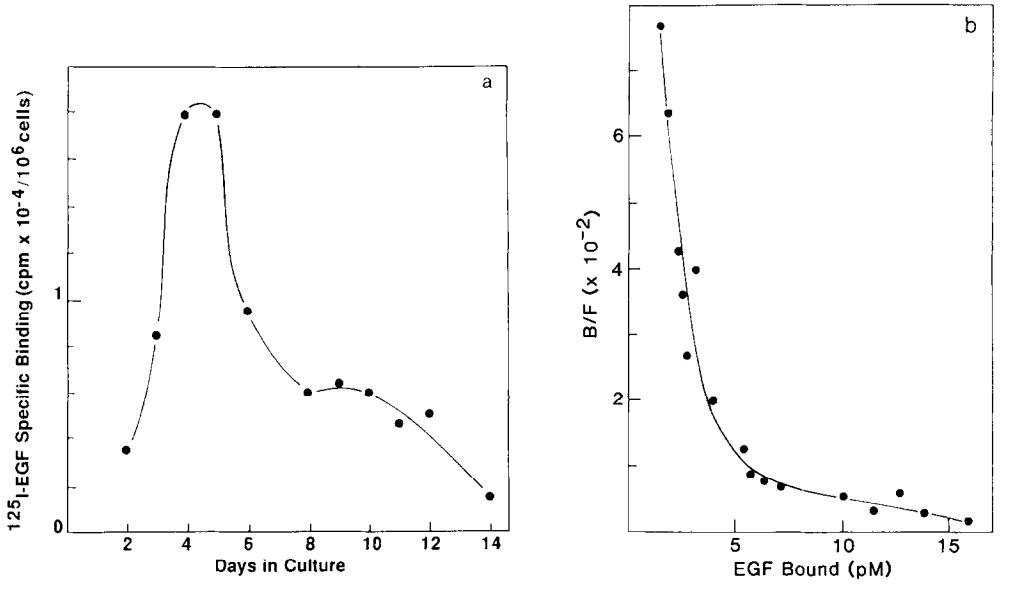

Fig. 2. [^{125}I]EGF binding to adipocyte precursors.

a. Time-course of [^{125}I]EGF binding: Binding was performed as described in the Materials and Methods section in the presence of 10^5 cpm of [^{125}I]EGF (256 µCi/µg). Non-specific binding was determined by measuring [^{125}I]EGF binding on duplicate dishes in the presence of 1 µg/ml unlabeled EGF. Specific binding was calculated by subtracting the nonspecific binding to the total binding. Cell number was determined on duplicate dishes.

b. Scatchard analysis of [^{125}I]EGF binding to adipocyte precursors: Binding conditions were described in the Materials and Methods section.

adipose cells after they were differentiated. Scatchard representation of EGF binding to the undifferentiated adipocyte precursors was curvilinear (Figure 2b). This could correspond to the existence of two family of binding sites: high affinity sites 4,800 receptors per cell with a Kd value of 4 x 10^{-11} M and low affinity sites 22,000 receptors per cell, with a Kd of 1.4 x 10^{-9} M. These values are in accordance with the values determined for the binding of EGF to other types of cells (20-21). Interestingly, the teratoma-derived adipogenic cell line 1246 which can proliferate and undergo differentiation in defined medium (10) and is also inhibited by EGF presents two types of EGF receptors. For these cells, the decrease of EGF binding which is also observed when the cells differentiate corresponds to a decrease in receptor number without changes in their dissociation constants (Serrero, submitted to publication).

EGF effect on adipose differentiation is not limited to the inguinal fat pads. Our preliminary studies indicate that EGF blocks differentiation of adipocyte precursors isolated from epididymal fat pads. Moreover, the effect of EGF on primary cultures of adipocyte precursors has been observed using

other rat strains (Lewis and Wistar), with rat epididymal fat pads, and also with mice inguinal and epididymal fat pads (data not shown).

EGF has been known to be a potent mitogen and differentiation factor both in vivo and in vitro (21-23), whereas TGF-α has been shown to influence tissue function (17,24-25). This paper is the first demonstration of the effect of EGF and/or TGF-α as modulators of adipose tissue differentiation and of the existence of EGF binding sites on the surface of adipocyte precursors. Experiments are underway to examine the mechanisms by which EGF and TGF-α act on adipocyte precursors and determine their possible role during adipose tissue development in vivo.

ACKNOWLEDGEMENTS

I would like to thank Dr. Wallace McKeehan for the gift of acidic and basic FGFs, Mrs. Dianne Mills for expert technical assistance, Mrs. Marina LaDuke for illustrations and Mrs. Julie Lamb for preparation of the manuscript.

This work was supported by grants from The National Institute of Health 1-PO1 CA37589, from the Juvenile Diabetes Foundation #185221, and from the Council for Tobacco Research, Grant #2003.

REFERENCES

1. Morikawa, M., Nixon, T., and Green, H. (1982) Cell 29, 783-789.
2. Serrero, G. (1984) In Mammalian Cell Culture: The use of serum-free hormone-supplemented media. (J.P. Mather, ed.) pp. 53-75. Plenum Press, New York, London.
3. Ailhaud, G. (1982) Mol. Cell. Biochem. 49, 17-31.
4. Garbi-Chihi, J., Grimaldi, P., Torresani, J., Ailhaud. G., (1981) J. Receptor. Res. 2, 153-173.
5. William, J.H., Polakis, E. (1977) Biochem. Biophys. Res. Comm. 77, 175-186.
6. Ignotz, R.A., Massague, J. (1985) Proc. Natl. Acad. Sci. USA 82, 8530-8534.
7. Sparks, R.L., and Scott, R.E. (1986) Exp. Cell. Res. 165, 345-352.
8. Zezulak, K.M., and Green, H. (1986) Science 233, 551-553
9. Serrero, G. (1985) In Vitro Cell. and Dev. Biol. 21, 535-540.
10. Serrero, G., Khoo, J.C. (1982) Anal. Biochem. 120, 351-359.
11. Serrero, G. (1986) In Cellular Endocrinology: Hormonal Control of Embryonic and Cellular Differentiation (G. Serrero and J. Hayashi, eds.) Vol. 226, pp. 191-204. Alan R Liss, New York.
12. Serrero, G., and Mills, D. (1987) In Vitro Cell. Dev. Biol. 23, 63-66.
13. McKeehan, W.L., and Ham, R.G. (1976) J. Cell. Biol. 71, 727-734.
14. Wise, L.S., and Green, H. (1979) J. Biol. Chem. 254, 273-275.
15. Hunter, W.M., and Greenwood, F.C. (1962) Nature 194, 495-496.
16. Broad, T.E., Ham, R.G. (1983) Eur. J. Biochem. 135, 33-39.
17. Derynck, R. (1986) J. Cell. Biochem. 32, 293-304.
18. Marquardt, H., Hunkapiller, M.W., Hood, L.E., and Todaro, G.J. (1984) Science 223, 1079-1082.
19. Marquardt, H., Hunkapiller, M.W., Hood, L.E., Twardzik, D.R., DeLarco, J.E., Stephenson, J.R., and Todaro, G.J. (1983) Proc. Natl. Acad. Sci. USA 80, 4684-4688.

20. Brown, K.D., Blay, J., Irvine, R.F. Heslop, J.P., and Berridge, M.J. (1984) Biochem. Biophys. Res. Commun. 123, 377-384.
21. Carpenter, G., and Zendegui, J.G. (1986) Exp. Cell Res. 164, 1-10.
22. Cohen, S., and Taylor, J.M. (1974) Rec. Prog. Hormone Res. 30, 533-550.
23. Cohen, S., Carpenter, G., and Lembach, K.J. (1975) Adv. Metab. Dis. 8, 265-284.
24. Tashjian, A.H., Voelkel, E.F., Lazzaro, M., Singer, F., Roberts, A.B., Derynck, R., Winkler, M., and Levine, L. (1985) Proc. Natl. Acad. Sci. USA 82, 4535-4538.
25. Ibbotson, K.J., Harrod, J., Gowen, M., D'Souza, S., Smith, D.D., Winkler, M., Derynck, R., and Mundy, G.R. (1986) Proc. Natl. Acad. Sci. USA 83, 2228-2232.