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Regulation of transferrin receptor expression at the cell surface by insulin-like growth factors, epidermal growth factor and platelet-derived growth factor

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Addition of platelet-derived growth factor (PDGF), recombinant insulin-like growth factor I (rIGF-I) or epidermal growth factor (EGF) to BALB/c 3T3 fibroblasts causes a marked increase in the binding of [^{125}I]diferric transferrin to cell surface receptors. This effect is very rapid and is complete within 5 min. The effect of EGF is transient, with [^{125}I]diferric transferrin binding returning to control values within 25 min. In contrast, PDGF and rIGF-I cause a prolonged stimulation of [^{125}I]diferric transferrin binding that could be observed for up to 2 h. The increase in the binding of [^{125}I]diferric transferrin caused by growth factors was investigated by analysis of the binding isotherm. Epidermal growth factor, PDGF and rIGF-I were found to increase the cell surface expression of transferrin receptors rather than to alter the affinity of the transferrin receptors. This result was confirmed in human fibroblasts by the demonstration that EGF, PDGF and rIGF-I could stimulate the binding of a monoclonal antibody directed against the transferrin receptor (OKT9) to the cell surface. Furthermore, PDGF and rIGF-I stimulated the sustained uptake of [^{59}Fe]diferric transferrin by BALB/c 3T3 fibroblasts, while EGF transiently increased uptake. Thus the effect of these growth factors to increase the cell surface expression of the transferrin receptor appears to have an important physiological consequence.
Key words: diferric transferrin uptake/receptor regulation

Introduction

Transferrin is a serum protein that binds iron and is an essential requirement for the growth of cultured cells (Hutchings and Sato, 1978). The uptake of iron into cells is mediated by specific cell surface receptors that cycle between the plasma membrane and endosomal membranes. The transferrin receptor, together with bound diferric transferrin, is internalized via coated pits (Harding *et al.*, 1983; Hopkins, 1983; Hopkins and Trowbridge, 1983; Willingham *et al.*, 1984) and coated vesicles (Booth and Wilson, 1981; Pearse, 1982) and delivered to endosomes (Harding *et al.*, 1983; Hopkins, 1983; Hopkins and Trowbridge, 1983), where the low pH (Van Renswoude *et al.*, 1982; Dautry-Varsat *et al.*, 1983; Lamb *et al.*, 1983) causes the iron to be released from the transferrin (Aisen and Listowsky, 1980). Apotransferrin remains bound to the receptor and is recycled back to the cell surface, where it dissociates (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983), allowing the receptor to again bind diferric transferrin in order to complete the cycle by which iron is brought into the cell (Ciechanover *et al.*, 1983).

Recently it has been reported that the cycling of the transferrin receptor can be regulated by tumor-promoting phorbol diesters (Rovera *et al.*, 1982; Klausner *et al.*, 1984; May *et al.*, 1984). In the absence of tumor promoters the transferrin receptor cycles at a basal rate (Watts, 1985). Addition of the potent phorbol diester 4β-phorbol-12β-myristate-13α-acetate (PMA) to HL60 or K562 cells causes a rapid internalization of transferrin receptors so that the steady-state distribution is altered resulting in a decreased cell surface expression of the receptors (Rovera *et al.*, 1982; Klausner *et al.*, 1984; May *et al.*, 1984). In other cell types, PMA has been reported to cause externalization of the transferrin receptor (Buys *et al.*, 1984). The reason for the difference in the response of these cells to PMA is not understood but, taken together, the data do suggest that the cycling of the transferrin can be regulated. Indeed, very recently Wiley and Kaplan (1984) have reported that in human fibroblasts epidermal growth factor (EGF) causes a transient increase in the expression of the transferrin receptor at the cell surface. The purpose of the experiments presented here was to investigate whether the cell surface expression of the transferrin receptor in BALB/c 3T3 fibroblasts can be regulated by other purified growth factors. We confirm that EGF causes a rapid and transient stimulation of transferrin receptor expression at the cell surface. In addition we observed that insulin-like growth factor I (IGF-I) and platelet-derived growth factor (PDGF), which are potent mitogens for BALB/c 3T3 fibroblasts, both cause a prolonged stimulation of the expression of the transferrin receptor at the cell surface. We conclude that the cycling of the transferrin receptor is regulated by multiple growth factors in BALB/c 3T3 fibroblasts.

Results

The binding of [^{125}I]diferric transferrin to the cell surface of quiescent BALB/c 3T3 fibroblasts was investigated by incubation of the cells at 0°C to prevent internalization of ligand-receptor complexes. Under these conditions non-specific binding constituted ~20% of the total binding observed and >95% of the bound [^{125}I]diferric transferrin could be released from cell monolayers by washing the cells briefly at pH 3.0 (0°C) as described (Haigler *et al.*, 1980; Lamb *et al.*, 1983). We conclude from these results that [^{125}I]diferric transferrin binding assays carried out at 0°C can be used to monitor the binding of diferric transferrin to the cell surface of BALB/c 3T3 fibroblasts.

Figure 1 shows that incubation of BALB/c 3T3 fibroblasts with several highly purified growth factors (EGF, PDGF, insulin, rIGF-I or IGF-II) for 5 min at 37°C caused a marked increase in the cell surface binding of [^{125}I]diferric transferrin assayed subsequently at 0°C. The effect of EGF on BALB/c 3T3 cells is similar to that recently reported by Wiley and Kaplan (1984) in experiments using human fibroblasts. However, we observed that a similar stimulation of [^{125}I]diferric transferrin was also caused by incubation of the cells with PDGF, insulin or insulin-like growth factors (Figure 1).

The effect of PDGF to stimulate the cell-surface binding of [^{125}I]diferric transferrin to BALB/c 3T3 fibroblasts was observed at concentrations of PDGF as low as 20 pM and was maximal at about 350 pM (Figure 1A). A similar concentration

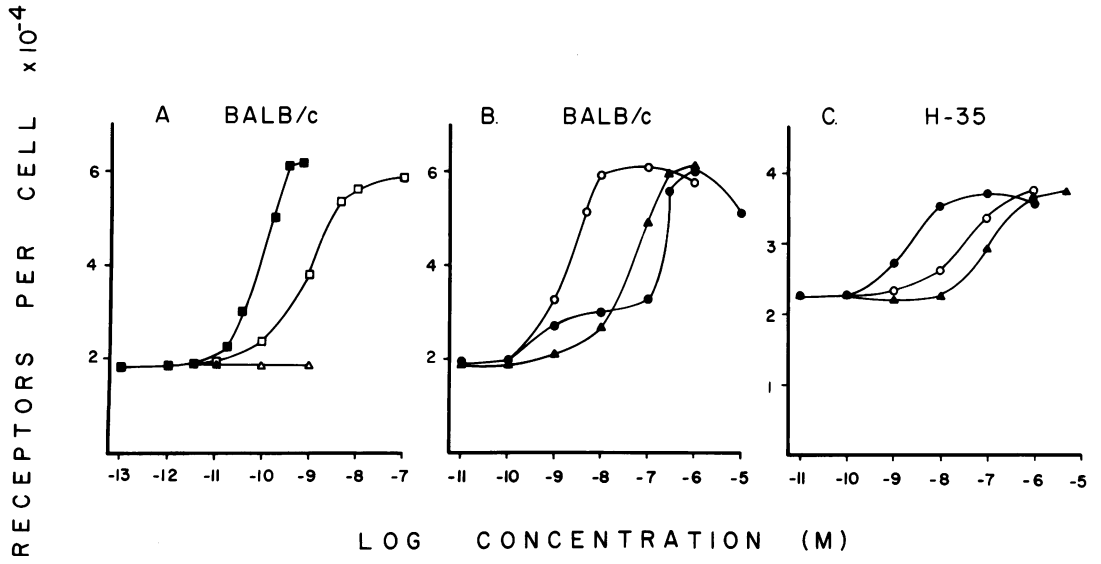

Fig. 1. Effect of growth factor concentration on the binding of [^{125}I]diferric transferrin to BALB/c 3T3 fibroblasts and H35 hepatoma cells. The specific binding of 5 nM [^{125}I]diferric transferrin was measured at 0°C following the treatment of post-confluent BALB/c 3T3 fibroblasts (**A**) and (**B**) or 2-day serum-starved sub-confluent H35 hepatoma cells (**C**) with different concentrations of purified growth factors for 5 min at 37°C. The growth factors used were PDGF (■), TGF-β (△), insulin (●), rIGF-I (○), IGF-II (▲) and EGF (□). The data presented were obtained from a single experiment. Similar results were obtained in three separate experiments.

dependence of PDGF action on mitogenesis and regulation of the EGF receptor has previously been reported (O'Keefe and Pledger, 1983). This action of PDGF was observed on BALB/c 3T3 fibroblasts, Swiss 3T3 fibroblasts and WI-38 human lung fibroblasts, but was not observed when PDGF was added to A431 epidermoid carcinoma cells (data not shown), which lack specific PDGF receptors (Heldin *et al.*, 1982). We conclude that PDGF at mitogenic concentrations stimulates the binding of [^{125}I]diferric transferrin to cells that express PDGF receptors. In contrast, another growth factor present in high levels in human platelets, TGF-β, was found to have no effect on the binding of [^{125}I]diferric transferrin to BALB/c 3T3 fibroblasts (Figure 1A), which express a high level (Massagué and Like, 1985) of functional (Massagué *et al.*, 1985) TGF-β receptors.

Insulin, rIGF-I and IGF-II were also observed to increase the binding of [^{125}I]diferric transferrin to the surface of BALB/c 3T3 cells (Figure 1B). The concentrations of rIGF-I, IGF-II and insulin required for the half-maximal response of the fibroblasts were 1, 30 and 150 nM respectively. The higher potency of rIGF-I compared to that of insulin raises the question of what receptor species mediates this response. The three ligands (insulin, rIGF-I and IGF-II) can bind to three distinct receptor species (insulin receptor, type I IGF receptor and type II IGF receptor) with markedly different affinities (for review see Czech, 1982). Previous studies have demonstrated by affinity cross-linking methods that BALB/c 3T3 fibroblasts express insulin, type I and type II IGF receptors (Massagué *et al.*, 1985). The order of potency of rIGF-I, IGF-II and insulin suggests very strongly that the type I IGF receptor is the receptor species that mediates this response based upon consideration of the known affinity constants of the three ligands for the type I IGF receptor (Czech, 1982).

In further experiments we investigated whether the potent effect of rIGF-I to stimulate [^{125}I]diferric transferrin binding was unique to BALB/c 3T3 fibroblasts or was also found in other cell lines. We observed that a low concentration (1 nM) of rIGF-I would stimulate the binding of [^{125}I]diferric transferrin to other rodent fibroblasts (Swiss 3T3, 3T3-L1 and NRK cells) as well as human fibroblasts (WI-38), human epidermoid carcinoma cells (A431) and human promyelocytic leukemia cells (HL60). We conclude that the regulation of the transferrin receptor by rIGF-I is exhibited by diverse cell lines from several species and is not restricted to BALB/c 3T3 fibroblasts.

Although it was apparent that rIGF-I was much more potent than insulin, a small effect of low concentrations of insulin (1 nM) was observed (Figure 1B). This suggests that in addition to the type I IGF receptor, the insulin receptor may also be capable of regulating the transferrin receptor. However, in experiments with BALB/c 3T3 cells it was not clear whether the effects of low concentrations of insulin were due to interaction with the insulin receptor or whether the insulin was binding to a high affinity type I IGF receptor population. Therefore, to address the question of whether the insulin receptor can regulate the transferrin receptor, we required a model system to investigate the insulin receptor in the absence of the type I IGF receptor. For this purpose we investigated the effect of insulin to regulate the binding of [^{125}I]diferric transferrin to H-35 hepatoma cells, which lack type I IGF receptors (Massagué *et al.*, 1982). It was observed that insulin could stimulate the binding of [^{125}I]diferric transferrin to the cell surface receptors of H-35 cells (Figure 1C). The H-35 cells were also responsive to rIGF-I and IGF-II, although these ligands were 10- and 100-fold less potent than insulin, respectively. The potency of rIGF-I and IGF-II in H-35 cells is consistent with the affinity of these ligands for the insulin receptor in H-35 cells (Czech, 1982; Massagué *et al.*, 1982). Thus, the insulin receptor probably mediates the effects of insulin, rIGF-I and IGF-II in H-35 cells. We conclude that the insulin receptor as well as the type I IGF receptor is capable of mediating the regulation of the transferrin receptor by insulin and insulin-like growth factors.

In further experiments we investigated the time course of the regulation of the expression of the transferrin receptor at the cell surface by rIGF-I, EGF and PDGF. Figure 2 shows that all of these growth factors rapidly increase the binding of [^{125}I]diferric transferrin to the surface of BALB/c 3T3 fibroblasts. Maximal effects in each case were observed between 2 and 5 min follow-

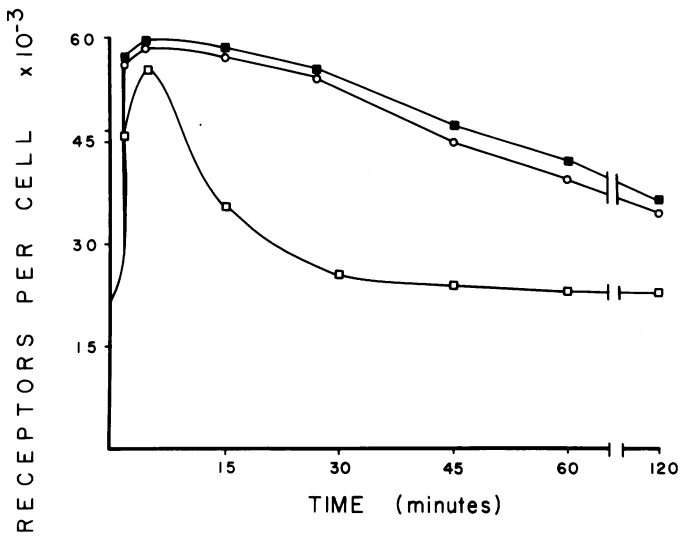

Fig. 2. Time course of the effect of growth factors to increase the cell surface expression of the transferrin receptor. Four-day post-confluent BALB/c 3T3 fibroblasts were incubated with 300 pM PDGF (■), 10 nM rIGF-I (○) or 10 nM EGF (□) for different times at 37°C. The cells were then cooled to 0°C and the specific binding of [^{125}I]diferric transferrin was measured. The data presented were obtained from a single experiment. Similar results were obtained in three separate experiments.

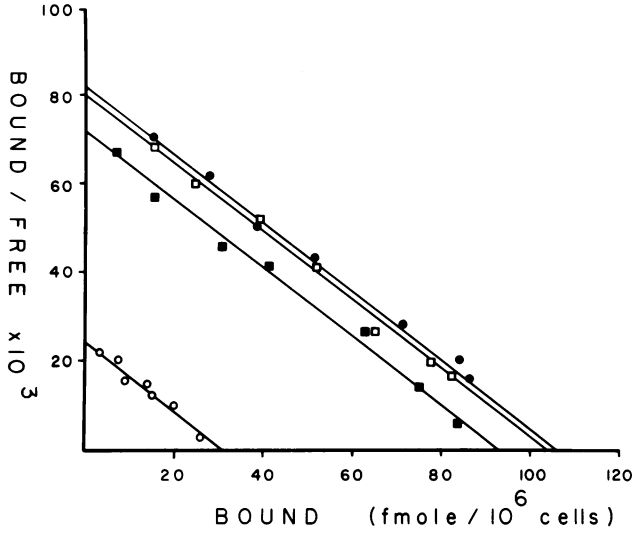

Fig. 3. Scatchard plot of the binding of [^{125}I]diferric transferrin to the surface of BALB/c 3T3 fibroblasts. Four-day post-confluent BALB/c 3T3 fibroblasts were incubated without (○) and with 300 pM PDGF (●), 10 nM rIGF-I (□) or 10 nM EGF (■) for 5 min at 37°C. The cells were then cooled for 0°C and the specific binding of different concentrations of [^{125}I]diferric transferrin were measured. The data are expressed by the method described by Scatchard (1949). The data presented were obtained from a single experiment. Similar results were obtained in two separate experiments.

ing the treatment of the cells with the purified growth factor. However, the time course of the effect observed after 5 min of treatment was markedly different when the cell surface expression of the transferrin receptor was increased by incubation of the cells with different growth factors. The action of EGF was found to be transient and very little effect of the EGF treatment was recorded 15–20 min following the incubation of the cells with EGF (Figure 2). In contrast, the effects of rIGF-I and PDGF could be observed after 60 min of incubation (Figure 2).

The increased binding of [^{125}I]diferric transferrin to BALB/c

Table I. Effect of growth factors on the binding of monoclonal anti-transferrin receptor antibody to the surface of WI-38 fibroblasts

Treatment	Specific binding (%)	
	Monoclonal antibody	Diferric transferrin
Control	100 ± 7	100 ± 5
PDGF	290 ± 18	320 ± 7
rIGF-I	295 ± 14	315 ± 12
EGF	278 ± 20	285 ± 9

Post-confluent WI-38 human fetal lung fibroblasts were incubated with 300 pM PDGF, 10 nM rIGF-I or 10 nM EGF for 5 min at 37°C. The cells were then cooled to 0°C. The specific binding of anti-human transferrin receptor monoclonal antibody ([^{125}I]OKT9) and [^{125}I]diferric transferrin were measured. The results are expressed relative to the binding of each ligand to control cells (100%) as the mean ± SEM of triplicate determinations. The data presented were obtained from a single experiment. Similar results were obtained in three separate experiments.

Table II. Effect of the state of confluency of BALB/c 3T3 fibroblasts on the surface expression of the transferrin receptor

Treatment	Transferrin bound (%)			
	Post confluent		Sub confluent	
	Control	Serum starved	Control	Serum starved
Control	100 ± 2	100 ± 1	100 ± 3	100 ± 2
PDGF	294 ± 14	319 ± 4	136 ± 13	330 ± 15
rIGF-I	305 ± 8	310 ± 6	171 ± 14	321 ± 6
EGF	268 ± 9	289 ± 12	179 ± 5	198 ± 8

BALB/c 3T3 fibroblasts were seeded in 16-mm wells and were used at a density of 50 000 cells/well or 4 days after confluence. Some of the cells were serum-starved by incubation for 30 h in medium containing 0.1% calf serum. The cells were treated with and without 300 pM PDGF, 10 nM rIGF-I or 10 nM EGF for 5 min at 37°C. The cells were then cooled to 0°C and the specific binding of 5 nM [^{125}I]diferric transferrin was measured. Non-specific binding was estimated in incubations containing a 100-fold excess of diferric transferrin. The results are expressed relative to the specific binding of [^{125}I]diferric transferrin observed to untreated (control) cultures (100%) as the mean ± SEM of triplicate determinations. The binding of [^{125}I]diferric transferrin to untreated cultures was estimated to be 1.8×10^4, 1.6×10^4, 4.5×10^4 and 2.5×10^4 molecules/cell for post-confluent cells, serum-starved post-confluent cells, sub-confluent cells and serum-starved sub-confluent cells respectively. The data presented were obtained from a single experiment. Similar results were obtained in three separate experiments.

3T3 fibroblasts that occurs after stimulation of the cells with EGF, rIGF-I or PDGF could be due to either a change in the affinity of the transferrin receptor or to a change in the number of transferrin receptors expressed at the cell surface. To distinguish between these two possibilities, we investigated the [^{125}I]diferric transferrin-binding isotherm of cells treated with or without growth factors. The data obtained are presented by the method of Scatchard (1949) in Figure 3. EGF, rIGF-I and PDGF were found to have little effect on the affinity of the transferrin receptor. However, EGF, rIGF-I and PDGF were found to increase the B_{max} from 31 fmol/10^6 cells to 93, 106 and 104 fmol/10^6 cells respectively (Figure 3). In order to confirm the conclusion that EGF, rIGF-I and PDGF cause an increase in the cell surface expression of the transferrin receptor, we investigated this action by an independent technique. The cell surface expression of the transferrin receptor was analyzed directly by measuring the binding of the monoclonal antibody OKT9 to WI-38 human fetal lung fibroblasts (Table I). Pre-teatment of the fibroblasts with EGF, rIGF-I or PDGF resulted in an increase in the specific binding of the anti-human transferrin receptor antibody to the

Table III. Effect of temperature on the response of BALB/c 3T3 fibroblasts to purified growth factors

Temperature (°C)	Transferrin bound (%)			
	Control	PDGF	rIGF-I	EGF
37	100 ± 1	340 ± 13	331 ± 12	284 ± 9
22	105 ± 4	259 ± 4	279 ± 8	232 ± 9
10	103 ± 3	136 ± 3	114 ± 3	107 ± 4
0	94 ± 3	97 ± 6	96 ± 6	99 ± 5

BALB/c 3T3 fibroblasts were seeded in 16-mm wells and grown to confluence. Four days after confluence the cells were used for the experiment. The cells were washed and incubated for 30 min at 37, 22, 10 or 0°C and then treated with 300 pM PDGF, 10 nM rIGF-I or 10 nM EGF for 5 min. All the cells were then cooled to 0°C and the specific binding of 5 nM [^{125}I]diferric transferrin was measured. Non-specific binding was estimated in incubations containing a 500-fold excess of diferric transferrin. The results are expressed relative to the specific binding of [^{125}I]diferric transferrin to control cells incubated for 30 min at 37°C (100%) as the mean ± SEM of triplicate determinations. The data presented were obtained from a single experiment. Similar results were obtained in three separate experiments.

Table IV. Effect of treating BALB/c 3T3 fibroblasts with purified growth factors or combinations of growth factors

Treatment	[^{125}I]Diferric transferrin bound (%)		
	5 min	30 min	60 min
Control	100 ± 2	106 ± 3	101 ± 2
EGF	275 ± 14	112 ± 3	105 ± 4
PDGF	310 ± 7	250 ± 9	206 ± 13
IGF-I	321 ± 10	231 ± 3	178 ± 9
PDGF and IGF-I	378 ± 9	332 ± 9	234 ± 12
PDGF and EGF	359 ± 8	297 ± 12	186 ± 13
IGF-I and EGF	325 ± 7	243 ± 6	183 ± 8

BALB/c 3T3 fibroblasts were seeded in 16-mm wells and were used 4 days after they had grown to confluence. The cells were washed, incubated at 37°C for 30 min and then treated with 300 pM PDGF, 10 nM rIGF-I or 10 nM EGF for 5, 30 or 60 min. The cells were then cooled to 0°C and the specific binding of 5 nM [^{125}I]diferric transferrin was measured. Non-specific binding was estimated in incubations containing a 100-fold excess of diferric transferrin. The results are expressed relative to the specific binding of [^{125}I]diferric transferrin to control cells (100%) as the mean ± SEM of triplicate determinations. The data presented were obtained from a single experiment. Similar results were obtained in three separate experiments.

cells (Table I). We conclude that EGF, rIGF-I and PDGF increase the cell surface expression of transferrin receptors.

To gain some insight into the mechanism of the action of EGF, rIGF-I and PDGF to stimulate the binding of [^{125}I]diferric transferrin to BALB/c 3T3 fibroblasts, we investigated the effect of temperature on this process. At 37°C EGF, rIGF-I and PDGF were found to increase markedly the binding of [^{125}I]diferric transferrin to BALB/c 3T3 fibroblasts. However, if the cells were incubated at 22 or 15°C, a sharp decrease in the responsiveness of the cells was observed. At 0°C, addition of growth factors to the cells did not affect the binding of [^{125}I]-diferric transferrin (Table II). The very high temperature dependence of the actions of EGF, rIGF-I and PDGF is consistent with the hypothesis that the increased expression of transferrin receptors at the cell surface is due to the translocation of intracellular transferrin receptors to the cell surface. As no effects of EGF, rIGF-I or PDGF are observed at low temperatures (Table II) it is possible to conclude that the action of these growth factors is not due to direct interaction of the growth factors with the transferrin receptor.

Table V. Effect of pre-treatment of BALB/c 3T3 fibroblasts with EGF on the subsequent response of the cells to EGF and other growth factors

First treatment	[^{125}I]Diferric transferrin bound (%) during the second treatment			
	Control	PDGF	rIGF-I	EGF
Control	100 ± 7	360 ± 14	295 ± 6	310 ± 15
EGF	110 ± 5	346 ± 12	287 ± 8	117 ± 3

BALB/c 3T3 fibroblasts were seeded in 16-mm wells and were used 4 days after they had grown to confluence. The cells were washed, incubated at 37°C for 30 min and then treated with or without 10 nM EGF for 10 min. The cells were then treated with or without 300 pM PDGF, 10 nM rIGF-I or 10 nM EGF for 5 min. The cells were cooled to 0°C and the specific binding of 5 nM [^{125}I]diferric transferrin was measured. Non-specific binding was estimated in incubations containing a 500-fold excess of diferric transferrin. The results are expressed relative to the binding to control cells that were not treated with growth factors (100%) as the mean ± SEM of triplicate determinations. The data presented were obtained from a single experiment. Similar results were obtained in three separate experiments.

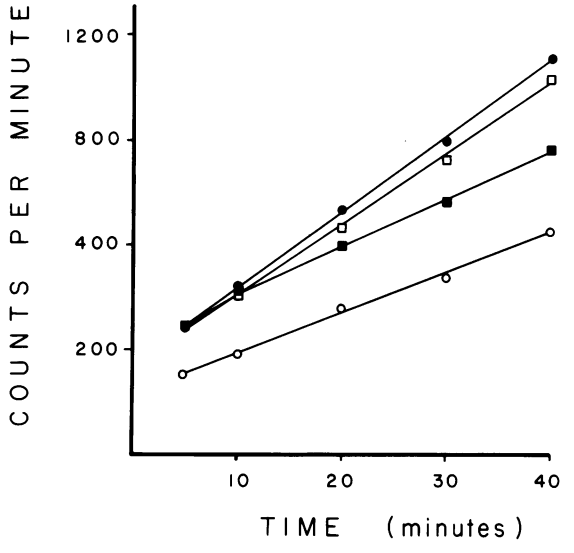

Fig. 4. Uptake of [^{59}Fe]diferric transferrin by BALB/c 3T3 fibroblasts. Post-confluent fibroblasts were incubated in 16-mm wells without (○) and with 10 nm EGF (■), 10 nM rIGF-I (□) or 300 pM PDGF (●) for 5 min at 22°C. [^{52}Fe]Diferric transferrin was added to a final concentration of 100 nM. At defined times, the monolayers were washed and solubilized, and the associated radioactivity was measured by scintillation counting. The data presented were obtained from a single experiment. Similar results were obtained in two separate experiments.

The interaction between EGF, IGF-I and PDGF in the regulation of the cell cycle of BALB/c 3T3 cells has been intensively studied (for review see Scher *et al.*, 1979). We therefore investigated the effects of these purified growth factors on the cell surface expression of the transferrin receptor using fibroblasts at different stages of growth. Post-confluent BALB/c 3T3 fibroblasts responded to EGF, rIGF-I and PDGF by increasing the cell surface expression of transferrin receptors (Table III). In contrast, sub-confluent cells were not observed to be very responsive to these growth factors unless the cells were first starved of serum for 30 h (Table III). We conclude that the stimulation of the cell surface expression of the transferrin receptor caused by EGF, rIGF-I and PDGF is greater in quiescent fibroblasts that have been growth-arrested by either confluence or serum starvation than in fibroblasts that are in the exponential growth phase.

In further experiments the interaction between EGF, rIGF-I

and PDGF was investigated by incubating post-confluent cultures of fibroblasts with either a single purified growth factor or with combinations of growth factors (Table IV). It was observed that there were no increased effects of EGF and rIGF-I when added together to the cells (Table IV). However, a significantly larger effect of PDGF was observed when it was added to cells together with EGF or rIGF-I (Table IV). These data indicate that there is an interaction between PDGF, rIGF-I and EGF in the regulation of the cell surface expression of the transferrin receptor. We therefore considered the possibility that the transient increase in the cell surface expression of the transferrin receptor, caused by the growth factors, could cause either homologous or heterologous densitization of the cells to subsequent stimulation by growth factors. The extremely transient effect of EGF on the cell surface expression of the transferrin receptor (Figure 2) was further investigated (Table V). Post-confluent fibroblasts were treated with or without 10 nM EGF for 30 min and then subsequently treated with or without 10 nM rIGF-I, 300 pM PDGF or 10 nM EGF for 5 min. It was observed that pre-treatment of the cells for 30 min with EGF inhibited the subsequent response of the cells to EGF, but did not affect the response of the cells to PDGF or rIGF-I (Table V).

The physiological significance of the regulation of the expression of the transferrin receptor at the cell surface was investigated. A primary function of the transferrin receptor is to bind diferric transferrin and mediate the accumulation of iron by cells. We therefore investigated the accumulation of iron by BALB/c 3T3 fibroblasts incubated with [^{59}Fe]diferric transferrin and treated with EGF, rIGF-I or PDGF. It was found that PDGF and rIGF-I caused a sustained increase in the rate of iron uptake (Figure 4). We conclude that the accumulation of iron by BALB/c 3T3 fibroblasts is acutely regulated by several growth factors which alter the expression of the transferrin receptor at the cell surface.

Discussion

Transferrin is a serum protein that is required for the growth of cultured cells in defined media (Hutchings and Sato, 1978). It has been recognized for several years that there is an association between the expression of the specific receptors for transferrin and cellular growth. Thus, quiescent lymphocytes do not express transferrin receptors, but if these cells are stimulated with antigen or lectin in the presence of interleukin II, active expression of transferrin receptors is observed (Galbraith and Galbraith, 1981; Sutherland *et al.*, 1981; Hamilton, 1982; Neckers and Crossman, 1983). Similarly it has been recently reported that interferon-α treatment of fibroblasts results in inhibition of growth and a decreased expression of transferrin receptors (Besancon *et al.*, 1985). In both of these examples, the changes in transferrin receptor expression are slow and several hours are required before any change in the level of receptor expression is observed. These changes are probably due to changes in the rate of synthesis or degradation of the receptor.

The data presented in this report provide further evidence of an association between cellular growth and the transferrin receptor. The potent mitogens PDGF, IGF-I and EGF were found to increase the expression of the transferrin receptor by BALB/c 3T3 fibroblasts at the cell surface. However, in marked contrast to the effects of antigen, lectin, interleukin-II and interferon, the effects of PDGF, IGF-I and EGF are observed to be extremely rapid. Maximal effects occurred within 5 min of treatment of the fibroblasts with these mitogens. The speed of this response indicates that the increase in the expression of the transferrin receptor at the cell surface cannot be accounted for by increased synthesis of the receptor. We propose the hypothesis that the mitogens cause the redistribution of transferrin receptors within the cell, resulting in an increased number of receptors in the plasma membrane and a decreased number of receptors in intracellular membrane compartments.

The action of PDGF, IGF-I and EGF to regulate the distribution of transferrin receptors between cell surface and intracellular membrane compartments bears some similarity to the regulation of the glucose transporter (Cushman and Wardzala, 1980; Suzuki and Kono, 1980) and type II IGF receptor (Oppenheimer *et al.*, 1983; Oka *et al.*, 1984; Wardzala *et al.*, 1984) by insulin in isolated fat cells. It has been reported that insulin causes an increase in the cell surface expression of the glucose transporter and type II IGF receptor, and a decrease in the number of intracellular glucose transporters and type II IGF receptors (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Oppenheimer *et al.*, 1983; Oka *et al.*, 1984; Wardzala *et al.*, 1984). We have observed that insulin will also cause an increase in the cell surface expression of the transferrin receptor in isolated fat cells (R.J.Davis, S.Corvera and M.P.Czech, in preparation). Thus, in the fat cell the transferrin receptor represents a third membrane component that rapidly redistributes between intracellular and cell surface membrane compartments following the treatment of fat cells with insulin.

A significant difference that exists between the regulation of the transferrin receptor in BALB/c 3T3 fibroblasts and isolated fat cells is the response to insulin. Fat cells are very responsive to insulin (data not shown), but the BALB/c 3T3 fibroblasts require very high concentrations of insulin before an increase in the binding of [^{125}I]diferric transferrin is observed (Figure 1A). However, BALB/c 3T3 fibroblasts are very responsive to IGF-I and the effects of insulin are probably largely due to the binding of the ligand to the type I IGF receptor rather than to the insulin receptor (Figure 1A). We screened several cell lines to investigate the relative potency of IGF-I and insulin to stimulate the binding of [^{125}I]diferric transferrin. It was found that rodent fibroblasts (BALB/c 3T3, Swiss 3T3, 3T3-L1 and NRK-49F), human epidermoid carcinoma cells (A431), human fibroblasts (WI-38) and human promyelocytic leukemia cells (HL60) all responded to low concentrations of rIGF-I but required high concentrations of insulin before a stimulation of [^{125}I]diferric transferrin binding was observed. Only H-35 hepatoma cells, which lack the type I IGF receptor (Massagué *et al.*, 1982), were found to respond better to low concentrations of insulin than IGF-I (Figure 1B). We conclude that both the insulin receptor and the type I IGF receptor can regulate the transferrin receptor. However, the relative roles of the insulin receptor and the type I IGF receptor depends on the cell line studied. In human and rodent fibroblasts, the type I IGF receptor plays a dominant role in the regulation of the transferrin receptor.

It has been reported that the transferrin receptor cycles between the cell surface and endosomal membranes independently of the presence of its ligand, transferrin (Watts, 1985). In the steady state, the rate of transferrin receptor endocytosis is equal to the rate of exocytosis. An increase in the number of receptors expressed at the cell surface can therefore be mediated by either a decrease in the rate of endocytosis or an increase in the rate of exocytosis. Identification of the site(s) of action of PDGF, IGF-I and EGF is an important goal for future progress to be made in the understanding of the mechanisms of transferrin receptor regulation. We conclude from the data presented in this report that the expression of the transferrin receptor at the cell surface of BALB/c 3T3 fibroblasts is regulated by multiple growth fac-

tors. The effects of EGF, rIGF-I or PDGF are too rapid to be accounted for by an increase in the rate of synthesis of the transferrin receptor. We propose the hypothesis that EGF, rIGF-I or PDGF cause the redistribution of intracellular transferrin receptors from an internal membrane compartment to the plasma membrane resulting in an increased expression of the transferrin receptor at the cell surface.

Materials and methods

Growth factors

Purified PDGF (Johnsson *et al.*, 1982) and TGF-β (Massagué and Like, 1985) were obtained from Drs C.H.Heldin and J.Massagué, respectively. EGF was purified as described (Savage and Cohen, 1972; Matrisian *et al.*, 1982). Porcine insulin was from Eli Lilly Co. IGF-II was purified from the conditioned medium of BRL-3A cells as described (Marquardt *et al.*, 1981). IGF-I produced by recombinant DNA technology (rIGF-I) was obtained from Amgen Corp. (Dr M.Peters). rIGF-I differs from IGF-I in that it contains an additional eight amino acids at the amino terminus (M K K Y W I P M) and a threonine substitution for methionine at position 59. Recent studies have demonstrated that rIGF-I and IGF-I bind to the type I IGF receptor with similar affinity (Peters *et al.*, 1985).

Cell culture

Swiss 3T3 fibroblasts, BALB/c 3T3 fibroblasts, 3T3L1 fibroblasts, hybridoma cells (OKT9), HL60 promyelocytic leukemia cells and WI-38 fibroblasts were purchased from the American Type Culture Collection. H-35 hepatoma cells were obtained from Dr G.Litwack (Temple University). A431 epidermoid carcinoma cells were obtained from Dr G.Todaro (Oncogen). NRK-49F cells were obtained from Dr J.DeLarco (National Cancer Institute).

Binding of [^{125}I]diferric transferrin to cell surface receptors

[^{125}I]Diferric transferrin (5–7 Ci/g) was prepared from transferrin (Calbiochem) as described (Van Renswoude *et al.*, 1982). Na^{125}I was obtained from New England Nuclear. Binding assays were performed on cells grown in 16-mm wells. The cells were washed with serum-free medium and incubated in medium containing 120 mM NaCl, 6 mM KCL, 1.2 mM $CaCl_2$, 1 mM $MgSO_4$ and 25 mM Hepes (pH 7.4) with 0.2% (w/v) bovine serum albumin. The cells were treated with and without purified growth factors at 37°C and then cooled to 0°C. [^{125}I]-Diferric transferrin was then added to the cells for 180 min at 0°C. The monolayers were then washed three times with cold medium and solubilized with 900 μl of 1.0 M NaOH. Radioactivity was quantitated with a Beckman gamma counter. Non-specific binding was estimated in incubations with a 100-fold excess of diferric transferrin.

Binding of [^{125}I]monoclonal anti-transferrin receptor antibody (OKT 9) to cell surface receptors

The monoclonal antibody OKT9 was purified from ascites fluid by ammonium sulfate precipitation, DEAE-Sephacel chromatography and chromatography on Sephacryl-S200. The antibody was iodinated as described (Klausner *et al.*, 1984) to a specific activity of 0.5–1.5 Ci/g. The binding of the antibody to the cell-surface receptors of WI-38 fibroblasts was measured in 16-mm wells. The cells were incubated in 120 mM NaCl, 6 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgSO_4$ and 25 mM Hepes (pH 7.4) with 0.2% (w/v) bovine serum albumin. The cells were treated with or without purified growth factors at 37°C and then cooled to 0°C. [^{125}I]OKT9 (2 μg/ml) was added to the cells for 180 min at 0°C. The monolayers were then washed three times with cold medium and solubilized with 900 μl of 1.0 M NaOH. Radioactivity was quantitated with a Beckman gamma counter. Non-specific binding of [^{125}I]OKT9 was estimated in incubations containing 0.2 mg/ml OKT9 antibody.

Uptake of [^{59}Fe]diferric transferrin by BALB/c 3T3 fibroblasts

[^{59}Fe]Diferric transferrin (32 000 c.p.m./μg) was prepared from transferrin (Calbiochem) and $^{59}FeCl_3$ (New England Nuclear) as described (Van Renswoude *et al.*, 1982). Post-confluent fibroblasts were washed and incubated in 120 mM NaCl, 6 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgSO_4$ and 25 mM Hepes (pH 7.4) with 0.2% (w/v) bovine serum albumin for 30 min at 22°C. The cells were then treated with and without growth factors for 5 min. Subsequently, [^{59}Fe]differic transferrin was added to a final concentration of 100 nM. At defined times, the monolayers were washed with cold medium and the cells were solubilized in 0.5 ml of 0.5% $NaDodSO_4$, 20 mM nitrilotriacetate (pH 6.4). The solubilized cells were mixed with 4 ml of Optifluor (Packard) and the associated radioactivity was measured in a Beckman scintillation counter.

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