Cell Surface Fibroblast Growth Factor and Epidermal Growth Factor Receptors Are Permanently Lost during Skeletal Muscle Terminal Differentiation in Culture

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Abstract. One characteristic of skeletal muscle differentiation is the conversion of proliferating cells to a population that is irreversibly postmitotic. This developmental change can be induced in vitro by depriving the cultures of specific mitogens such as fibroblast growth factor (FGF). Analysis of cell surface FGF receptor (FGFR) in several adult mouse muscle cell lines and epidermal growth factor receptor (EGFR) in mouse MM14 cells reveals a correlation between receptor loss and the acquisition of a postmitotic phenotype. Quiescent MM14 cells, mitogen-depleted, differentiation-defective MM14 cells, and differentiated BC3H1 muscle cells (a line that fails to become postmitotic upon differentiation) retained their cell surface FGFR. These results indicate that FGFR loss is not associated with either reversible cessation of muscle cell proliferation or biochemical differentiation and thus, further support a correlation between receptor loss and acquisition of a postmitotic phenotype. Comparison of the kinetics for growth factor receptor loss and for commitment of MM14 cells to a postmitotic phenotype reveals that FGFR rises transiently from ∼700 receptors/cell to a maximum of ∼2,000 receptors/cell 12 h after FGF removal, when at the same time, >95% of the cells are postmitotic. FGFR levels then decline to undetectable levels by 24 h after FGF removal. During the interval in which FGFR increases and then disappears there is no change in its affinity for FGF. The transient increase in growth factor receptors appears to be due to a decrease in ligand-mediated internalization because EGFR, which undergoes an immediate decline when cultures are deprived of FGF (Lim, R. W., and S. D. Hauschka. 1984. *J. Cell Biol.* 98:739–747), exhibits a similar transient rise when cultures are grown in media containing both EGF and FGF before switching the cells to media without these added factors. These results indicate that the loss of certain growth factor receptors is a specific phenotype acquired during skeletal muscle differentiation, but they do not resolve whether regulation of FGFR number is causal for initiation of the postmitotic phenotype. A general model is presented in the discussion.

As a model system to study the loss of proliferative capacity that accompanies terminal differentiation, we investigated the mechanisms involved in the irreversible conversion of proliferating skeletal muscle myoblasts to a postmitotic phenotype. This transition, which occurs both in vivo (Snow, 1977, 1978) and in culture (Konigsberg, 1975; Nadal-Ginard, 1978; Hsu et al., 1979), is known to be influenced by components in the growth medium (Yaffe et al., 1971; Konigsberg, 1971). Impure fibroblast growth factor (FGF)[1] preparations, for instance, were shown to promote mouse and bovine myoblast proliferation, and to repress skeletal muscle terminal differentiation (Linkhart et al., 1980, 1981; Gospodarowicz et al., 1976). More recently, we have shown in mouse MM14 myoblasts that pure preparations of both acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) repress the onset of skeletal muscle differentiation, thereby preventing the acquisition of a permanent postmitotic phenotype, transcriptional induction of skeletal muscle specific genes, and cell fusion (Clegg et al., 1987). FGF-dependent repression of myogenic differentiation is not limited to murine skeletal muscle cell lines. Primary human skeletal muscle myoblasts exhibit FGF-dependent and FGF-independent colony forming types (Hauschka, S. D., and J. Seed, unpublished data). Although the FGF-independent clones do not require added FGF for growth, their differentiation is delayed in the presence of FGF. A similar response has been observed for primary chick skeletal muscle myoblasts (Kardami et al., 1985*a*; Seed and Hauschka, 1988).

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[1] *Abbreviations used in this paper*: aFGF and bFGF, acidic and basic fibroblast growth factors, respectively; DM, deficient medium (includes Ham's F-10C plus 5% horse serum and 1 μM insulin; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FM, complete medium (includes Ham's F-10C plus 15% horse serum); HS, horse serum; MHC, myosin heavy chain; TGF-β, transforming growth factor-β.

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Since our previous studies indicate that FGF plays an important role in the regulation of skeletal muscle differentiation, and since terminal differentiation of MM14 cells is accompanied by a permanent loss of epidermal growth factor receptor (EGFR) (Lim and Hauschka, 1984*a*), we hypothesized that the postmitotic phenotype is a consequence of growth factor receptor loss. This model was speculative since MM14 myoblasts do not exhibit a proliferative response to EGF even though they possess ~10,000 EGFR per cell. With the subsequent availability of ^{125}I-aFGF and identification of the cross-linked ^{125}I-aFGF–fibroblast growth factor receptor (FGFR) complex in MM14 cells (Olwin and Hauschka, 1986), it became feasible to test the mitogen receptor-loss model with respect to FGFR.

In this report, we examine growth factor receptor behavior to determine how cell surface receptor levels change during the differentiation of skeletal and smooth muscle cell types; and to determine the temporal relationship between changes in FGFR and EGFR levels and the kinetics for acquisition of a postmitotic phenotype in mitogen-depleted skeletal muscle cultures.

Materials and Methods

Tissue Culture

Murine MM14 cells (Hauschka et al., 1979; Linkhart et al., 1981) and C2C12 cells (derived by Yaffe and Saxel, 1977; subcloned by Blau et al., 1985; and kindly provided by Dr. A. Lassar, Hutchinson Cancer Research Center, Seattle, WA) were grown on gelatin-coated culture dishes in Ham's F-10C (F-10 supplemented with 0.8 mM Ca^{2+}), increasing concentrations of a mixture of aFGF and bFGF from 2–10 ng/ml as the culture density increased from 1–2 × 10^5–1 × 10^6 cells per 100-mm dish, 1% antibiotics (10,000 U/ml penicillin G, 0.5 mg/ml streptomycin sulfate, 0.05 mg/ml gentamicin), and 15% horse serum (HS). BC3H1-A1 cells were re-subcloned by us from BC3H1 cells (No. 1443; American Type Culture Collection, Rockville, MD) to yield a line with much more homogeneous differentiation. The cells were grown in DME containing 20% FCS and 4 ng/ml FGF. Complete medium (FM) includes Ham's F-10C plus 15% HS and deficient medium (DM) includes Ham's F-10C plus 5% HS and 1 μM insulin (included to prevent myotube degeneration).

Purification and Iodination of aFGF

These procedures were carried out as described (Olwin and Hauschka, 1986) with the following modification. Heparin–Sepharose-purified aFGF was further purified to remove trace contaminants by FPLC on a mono-S column (Pharmacia, Inc., Piscataway, NJ) and eluted with a 0.1–1.0 M NaCl gradient in 20 mM Hepes, pH 7.4.

Iodination of EGF

Receptor grade EGF (Sigma Chemical Co., St. Louis, MO) was incubated with 2.0 mCi Na ^{125}I and 60 μg of chloramine T for 5 min at 22°C in 0.5 M sodium phosphate, pH 7.0. Sodium bisulfite (120 μg) was added at 5 min and the products were incubated an additional 5 min before separating free ^{125}I from ^{125}I-EGF by chromatography on Sephadex G-15 (preequilibrated in 20 mM Hepes pH 7.4, 150 mM NaCl, and 0.2% BSA). ^{125}I-EGF specific activity was ~1.4 × 10^6 dpm/pmol.

Assays

Postmitotic cells. Postmitotic cells were detected by their inability to form colonies when passaged and replated at low density (1,000 cells per 100-mm dish) in FM containing 6 ng/ml FGF. Cells were grown for 4 d and the number of macroscopic colonies (eight or more cells) counted. Percent commitment was defined as: 100 × [1 − (*n* macroscopic clones/*n* clones from control plate before FGF removal)].

Myosin Heavy Chain (MHC). Cultures were rinsed twice with PBS, fixed for 1 min (20:2:1, 70% ethanol/formalin/glacial acetic acid) at 4°C and stored in PBS at 4°C. MHC was detected by incubating plates with the MF-20 monoclonal antibody (Bader et al., 1982) diluted with 1% HS in 50 mM Tris-Cl, pH 7.4, 100 mM NaCl for 1 h. Plates were rinsed and stained using an avidin-biotin-horseradish peroxidase procedure (Vector Laboratories, Inc., Burlingame, CA).

Single Cell Autoradiography. Cultures were rinsed twice with Ham's F-10C containing 25 mM Hepes, pH 7.4 and incubated for 1 h with 100 pM ^{125}I-aFGF in Ham's F-10C containing 25 mM Hepes, pH 7.4 and 0.2% BSA (binding buffer) for 1 h at 22°C, rinsed four times with ice-cold PBS, and fixed with 2% gluteraldehyde in PBS at 4°C for 2 min. The dishes were rinsed 10 times with glass-distilled H_2O, air dried, coated with Kodak NTB2 liquid emulsion, and exposed for 1–2 wk at 4°C. The plates were developed according to the manufacturer's instructions.

Binding and Cross-linking of ^{125}I-aFGF. The binding and cross-linking of ^{125}I-aFGF to intact cell cultures was performed as described (Olwin and Hauschka, 1986). Briefly, for equilibrium binding, intact cells were plated at 1 × 10^6 cells on 35-mm dishes and incubated for 2 h in FM with or without FGF. The cultures were rinsed twice in Ham's F10C containing 25 mM Hepes, pH 7.4 and 0.2% BSA. ^{125}I-aFGF was then added without or with a 100-fold M excess of aFGF to determine nonspecific binding and the cultures incubated on a rocker platform at 30 cycles/min for 1 h at 22°C or 3 h at 10°C. Cultures were then rinsed three times in PBS containing 2 mM $MgCl_2$ and 0.2% BSA, and the bound ^{125}I-aFGF solubilized in PBS containing 1% Triton X-100.

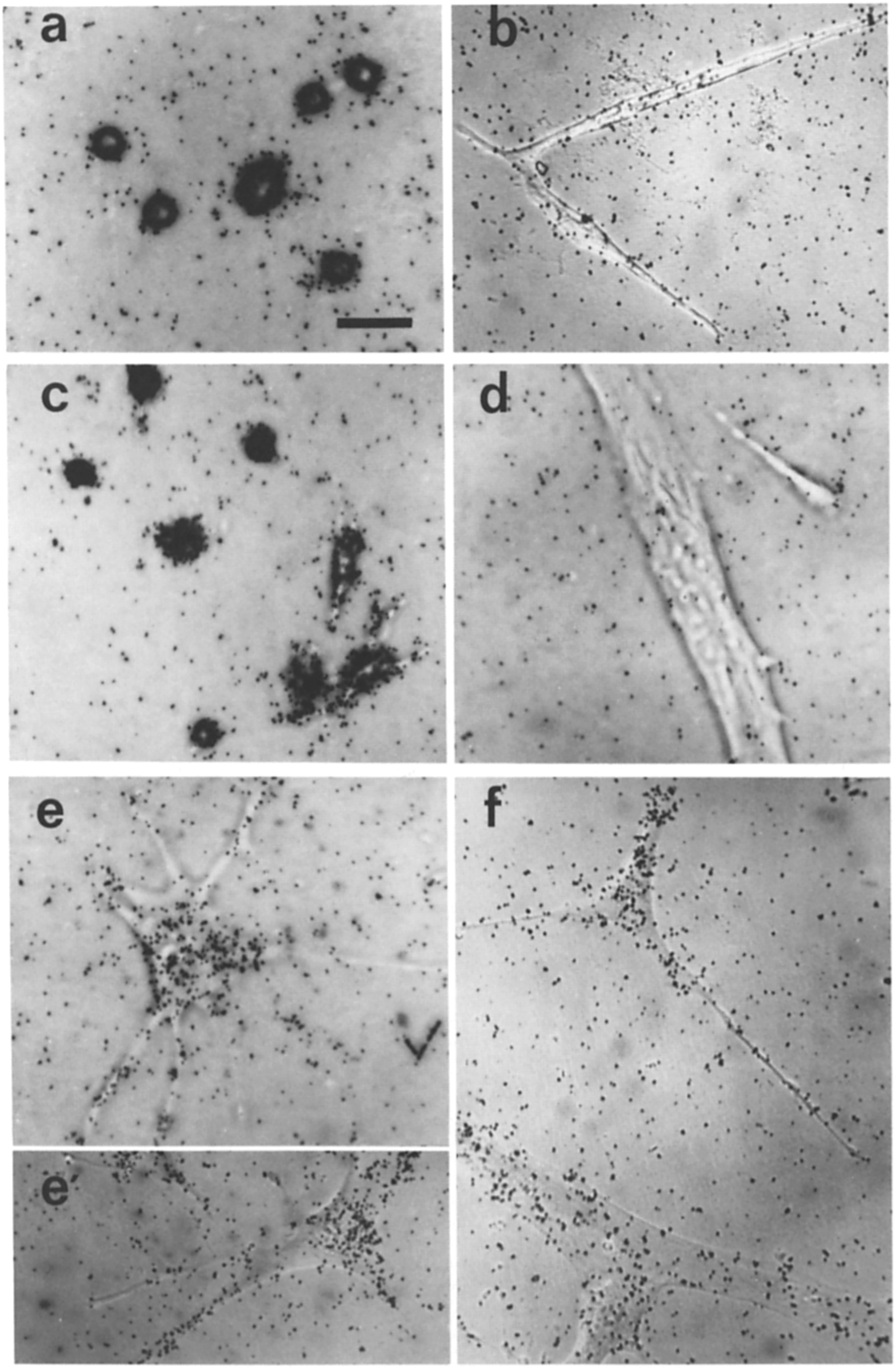
Cross-linking of ^{125}I-EGF. ^{125}I-EGF (8.0 nM) was incubated with intact MM14 cultures for 1 h at 22°C in binding buffer. The dishes were then rinsed three times with ice-cold PBS and incubated with 4 mM ethylene carbodiimide (Pierce Chemical Co., Rockville, IL) in PBS (pH 6.5) for 30 min at 22°C, rinsed twice with ice-cold PBS (pH 7.4), and processed as previously described for ^{125}I-aFGF–FGFR detection by SDS-PAGE and autoradiography (Olwin and Hauschka, 1986).

Results

FGF Binding to Proliferating and Differentiated Muscle Cultures

To detect cell surface FGFR on proliferating and differentiated muscle cells, ^{125}I-aFGF binding sites were visualized using single cell autoradiography. The results indicate that proliferating skeletal muscle cells (MM14, C2C12), as well as smooth muscle cells (BC3H1-A1) exhibit specific FGF binding (Fig. 1, *a*, *c*, and *e*). In contrast, terminally differentiated MM14 and C2C12 cells exhibit a total loss of FGF binding, irrespective of whether they are mononucleated or multinucleated (Fig. 1, *b* and *d*), whereas differentiated BC3H1-A1 cells, a brain tumor–derived smooth muscle cell line that does not become postmitotic upon differentiation (Schubert et al., 1974; Lathrop et al., 1985*a*), maintains high levels of ^{125}I-aFGF binding (Fig. 1 *f*). Interestingly,

Figure 1. ^{125}I-aFGF binding to MM14, C2C12, and BC3H1-A1 cells before and after differentiation. Proliferating myoblasts were cultured in FM plus 6 ng/ml FGF (MM14), DME plus 20% FCS and 6 ng/ml FGF (C2C12 and BC3H1-A1) for 48 h. Differentiated cultures were grown in the same medium for 48 h then rinsed three times with saline G and incubated in Ham's F-10C containing 1 μM insulin plus 5% HS, DME plus 1% HS, and DME plus 0.5% FCS for MM14, C2C12, and BC3H1-A1 cultures, respectively. Cultures were rinsed, incubated with 100 pM ^{125}I-aFGF, fixed, and processed for autoradiography as described in Materials and Methods. Differentiated MM14, C2C12, and BC3H1-A1 cultures were maintained in their mitogen-poor medium for 24, 48 h, and 4 d, respectively. In this period after mitogen withdrawal, MM14, C2C12, and BC3H1-A1 cells exhibited 100, 75, and 85% MHC-positive cells, respectively. Photographs are bright field images of proliferating MM14, C2C12, and BC3H1-A1 (*a*, *c*, *e*, and *e'*) and differentiated MM14, C2C12, and BC3H1-A1 (*b*, *d*, and *f*) cultures, respectively. Bar, 30 μm.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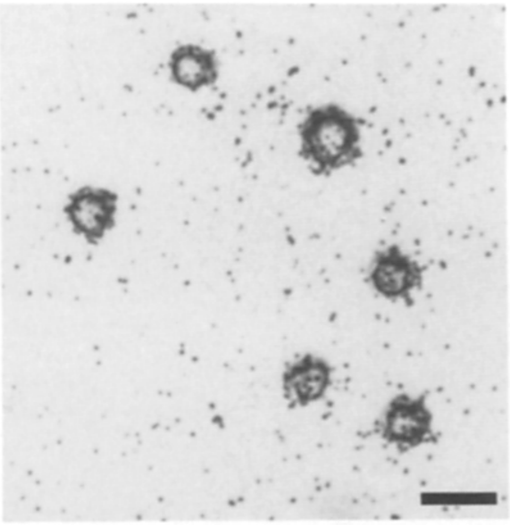

Figure 2. Quiescent MM14 myoblasts retain cell surface FGFR. MM14 myoblasts were grown 12 h in FM plus FGF then rinsed and switched to Ham's F-10C including 1% HS and 1 μM insulin in the presence of 10 ng/ml FGF for 24 h. At this time one set of parallel cultures was incubated with 100 pM ^{125}I-aFGF for 1 h at 22°C, fixed, and processed for autoradiography as described in Materials and Methods. The other was rinsed, incubated for 30 min with 3 μCi/ml [^{3}H]thymidine, fixed, and processed for MHC detection and autoradiography. Less than 8 and 15% of the cells were [^{3}H]thymidine positive and MHC positive, respectively. By comparison, proliferating MM14 cells are 60% [^{3}H]thymidine positive and <1% MHC positive while differentiated cells are <1% [^{3}H]thymidine positive and >99% MHC positive. Bar, 30 μm.

the rat L6 skeletal muscle cell line does not exhibit ^{125}I-aFGF binding in either proliferating or differentiated cultures (not shown).

FGFR Loss Is Specifically Associated with the Postmitotic Phenotype of Skeletal Muscle Cells

If FGFR loss is associated with the skeletal muscle postmitotic phenotype and not simply with cessation of cell proliferation, then FGFR should be present in quiescent, myoblast cultures. This could be examined in MM14 myoblasts that were maintained in low concentrations of HS and high concentrations of FGF. Under these conditions MM14 cells withdraw from the cell cycle, but do not differentiate; they will replicate once serum is restored, demonstrating that they have not committed to a permanent postmitotic phenotype (Clegg et al., 1987). When quiescent cultures were examined for FGFR by single cell autoradiography (Fig. 2) or by cross-linking ^{125}I-aFGF to intact cells (not shown), they retained high levels of cell surface FGFR. However, if MM14 cells were maintained for 24 h in 1 or 5% HS without FGF they exhibited no detectable cell surface FGFR (Figs. 1 *b* and 4; Table I). A similar total loss of FGF binding was exhibited by C2C12 cells when they were deprived of serum mitogens for ≥48 h (Fig. 1, *a* and *d*). A further indication that FGFR loss is associated with the skeletal muscle postmitotic phenotype is seen from the behavior of differentiation-defective (DD-1) myoblasts (Lim and Hauschka, 1984*b*). This variant MM14 cell line behaves similarly to the parental MM14 cells by withdrawing from the cell cycle when both FGF and serum are depleted, but the cells do not become postmitotic and in addition, they do not lose their cell surface FGFR (Table I). Taken together, these results indicate that the loss of FGFR is associated with the skeletal muscle postmitotic phenotype and is not simply a consequence of a muscle cell's proliferative state.

Kinetic Analysis of FGFR Behavior during MM14 Terminal Differentiation

To determine the kinetics of FGFR loss during terminal differentiation, MM14 cell surface FGFR was analyzed by equilibrium binding (Fig. 3; Table I) and by cross-linking ^{125}I-aFGF to intact cells (Fig. 4). After 24 h of FGF withdrawal FGFR was undetectable; however, an unexpected increase in FGFR was revealed during the first 12 h after FGF removal. This result could represent a true increase in cell surface FGFR; alternatively it could be due to an increased affinity of the receptor for FGF, or to an artifact due to unlabeled FGF remaining after the switch to FGF-deficient medium; such residual FGF would be gradually degraded, but would initially dilute the ^{125}I-aFGF specific activity. The latter two alternatives appear to be eliminated by the following observations. First, analysis of the equilibrium binding data reveals no significant change in the K_d for ^{125}I-

Table I. FGFR Behavior in MM14 and MM14 DD-1 Cells

FGF deprivation[*]	Cell type[‡]	K_d[§]	FGFR per cell[§]	
h		*pM*		
0	MM14	8 ± 3	700 ± 250[‖]	(3)
12	MM14	7 ± 1	2,000 ± 500	(3)
24	MM14	nm[¶]	0[¶]	(4)
0	MM14 DD-1	12	10,500	(2)
24	MM14 DD-1	11	12,000	(2)
60	MM14 DD-1	11	9,000	(2)

[*] Cells were maintained in FM plus 10 ng/ml FGF. FGF was removed by rinsing three times with Puck's Saline G and cells were incubated in DM for the times indicated before equilibrium ^{125}I-aFGF binding.

[‡] MM14 DD-1 cells are a nondifferentiating MM14 variant that does not require FGF for proliferation (Lim and Hauschka, 1984*b*).

[§] Data for K_d and FGFR number per cell were obtained by Scatchard analysis of equilibrium binding data.

[‖] Mean and standard deviation. Numbers in parentheses refer to number of independent experimental determinations. Where two determinations were performed the mean is reported.

[¶] Not measurable (values for specific and nonspecific ^{125}I-aFGF binding were equal).

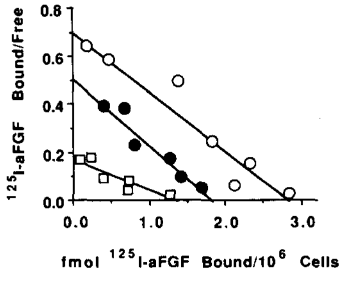

Figure 3. Equilibrium binding of ^{125}I-aFGF to intact differentiating MM14 cultures. MM14 myoblasts were grown in FM plus FGF (□) or in FM without FGF for 7 (●), and 12 h (○). Cultures were processed as described in Materials and Methods. The data were plotted according to Scatchard and normalized to 1 × 10^6 cells. On average 5 × 10^5 cells were on each 35-mm dish and the nonspecific binding did not exceed 20% of the total bound cpm at saturation. The data plotted are the mean of triplicate points from parallel cultures of a single experiment.

aFGF binding during FGF deprivation (Fig. 3 and Table I). Second, an independent analysis of the rinsing procedure indicates that >98% of the FGF initially present is removed before the addition of ^{125}I-aFGF. The residual low concentration of unlabeled FGF (<5 pM) is insufficient to compete significantly for binding of 100 pM ^{125}I-aFGF to intact cells (Olwin and Hauschka, 1986). Third, cross-linking MM14 cells with fivefold higher concentrations of ^{125}I-aFGF (500 pM) also revealed the transient two- to threefold increase in FGFR during the first 12 h of FGF withdrawal. The transient rise in FGFR before FGFR declines seems most readily explained by a cessation of rapid ligand-induced internalization upon FGF withdrawal while an existing pool of intracellular FGFR is continually inserted into the plasma membrane (see below).

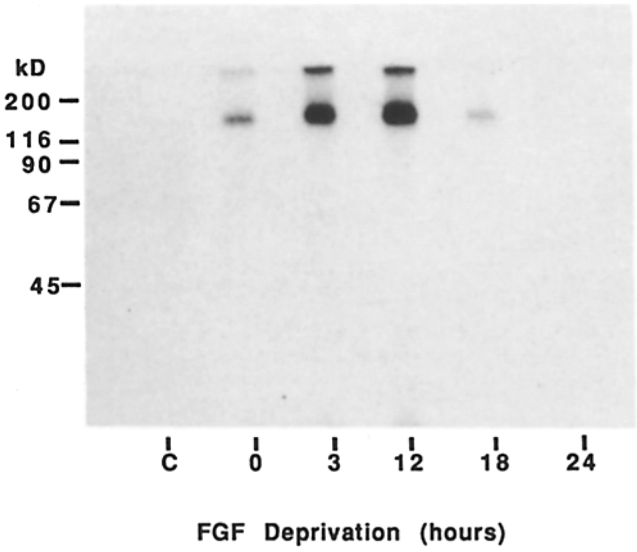

Figure 4. Cross-linking of ^{125}I-aFGF to differentiating MM14 cultures. MM14 cultures were grown in FM plus FGF or DM for the various times indicated. Cultures were then rinsed and incubated with ^{125}I-aFGF as described in Materials and Methods. Cross-linking, SDS-PAGE, and autoradiography were performed as described (Olwin and Hauschka, 1986). The film was exposed for 14 d at −70°C with a DuPont Cronex Intensifying Screen. The control lane (*C*) included 10 nM unlabeled aFGF during the ^{125}I-aFGF-incubation period. The uppermost bands represent higher order cross-linked material that did not enter the 7.5% SDS–polyacrylamide gel. The entire samples from identical numbers of cells (1 × 10^6) were run in each lane. The experiment was repeated three times with similar results.

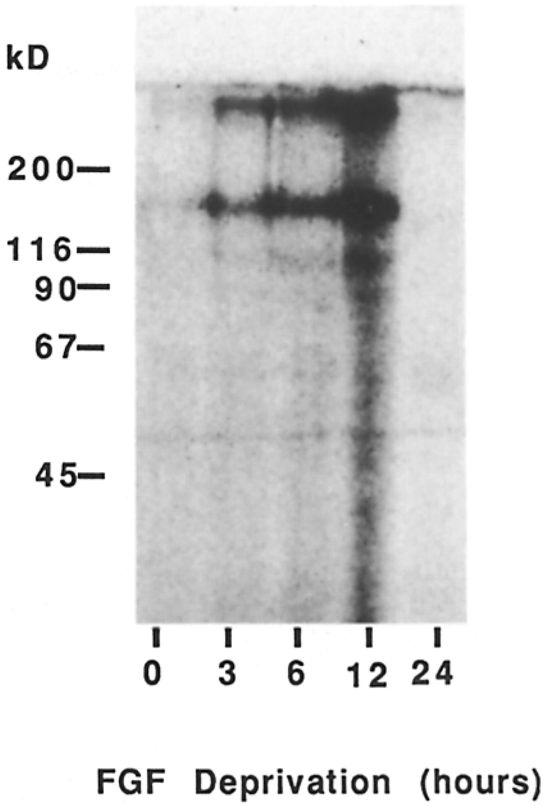

Figure 5. Cross-linking of ^{125}I-EGF to differentiating MM14 cultures. MM14 cultures were grown in FM plus FGF and 100 ng/ml EGF then switched to DM for the various times indicated and analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. 1 × 10^6 cells were cross-linked and the entire samples were run in each lane of the SDS-polyacrylamide gel. The ^{125}I-EGF–EGFR complex migrated at 175 kD and the uppermost bands represent higher order cross-linked material that did not enter the SDS-polyacrylamide gel. The experiment was repeated twice with similar results.

Analysis of EGFR Behavior during MM14 Terminal Differentiation

MM14 cells exhibit cell surface EGFR but do not respond mitogenically to EGF. Whether EGF has other effects on skeletal muscle cells is not known. In previous studies EGFR was examined in FGF-deprived, MM14 cultures as a model for growth factor receptor behavior during muscle differentiation (Lim and Hauschka, 1984*a*). Initial experiments examining EGFR loss during skeletal muscle differentiation suggested that a general loss of mitogen receptors might be a causal event for commitment to a postmitotic phenotype (Lim and Hauschka, 1984*a*). We have reexamined EGFR decline to determine whether the kinetics of its loss are consistent with a common EGFR and FGFR regulatory mechanism. In contrast to the earlier experimental protocol in which EGFR was measured in cultures that had not been exposed to EGF-containing medium before FGF removal, both EGF and FGF were included in the initial growth medium and then removed simultaneously. With this protocol EGFR, like FGFR, would be subjected to the absence of ligand-mediated internalization, thus allowing a more direct comparison of EGFR and FGFR behavior in FGF-deprived cultures (Fig. 4). Analysis of EGFR by cross-linking ^{125}I-EGF to intact MM14 cells at various times after EGF and FGF removal indicated both a transient increase and then a complete loss of EGFR, similar to that observed for FGFR (Fig. 5).

Kinetic Analysis of FGFR Loss versus the Kinetics of Commitment to a Postmitotic Phenotype

If FGFR loss is the causal event for irreversible acquisition ("commitment") of growth factor nonresponsiveness, then FGFR decline should either precede or parallel the kinetics of commitment to a postmitotic phenotype. In this experiment postmotitic cells were detected by their failure to form colonies when replated in FM including 6 ng/ml FGF at various times after FGF deprivation. A comparison of the kinetics for acquisition of a postmitotic phenotype and cell surface receptor loss (Figs. 3 and 4) in parallel cultures indicated that commitment to a postmitotic phenotype exceeds 50% 6 h after FGF removal, while the cell surface FGFR level has increased two- to threefold during the same interval (Fig. 6). At 12 h, when virtually all the cells have committed to a postmitotic phenotype, the cell surface FGFR level is still elevated; it does not decline until sometime after 12 h, becoming undetectable by 24 h. Similar FGFR behavior is observed whether the receptor quantitation is by chemical cross-linking or by equilibrium binding. From these results the cell surface FGFR levels detected during the first 12 h after FGF deprivation cannot be directly implicated as causing the postmitotic phenotype (see Discussion). However, since cell surface FGFR is absent by 24 h, this could be implicated as the primary mechanism responsible for maintenance of differentiated muscle cells in a postmitotic state.

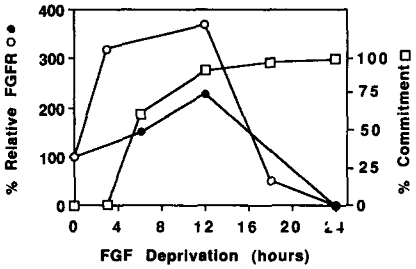

Figure 6. Comparison of the kinetics of FGFR loss and commitment to a postmitotic phenotype. The percent relative FGFR was determined from both cross-linked ^{125}I-aFGF and equilibrium binding experiments and is compared with the percent cells committed to a postmitotic phenotype after the same intervals of FGF deprivation. Cross-linked ^{125}I-aFGF-FGFR (●) was determined from a densitometric scan of the autoradiogram in Fig. 4, where 100% was the initial value of the ^{125}I-aFGF–FGFR complex at the time of FGF removal. Equilibrium binding values for FGFR (○) were determined from the data in Fig. 3, where 100% was the initial FGFR number per cell at the time of FGF removal. Percent commitment (□) was determined in parallel cultures by passaging cells at the indicated times after FGF withdrawal, replating the cells at low density (1,000 per 100-mm dish) and incubating in FM plus 10 ng/ml FGF for 4 d. The cultures were stained with hematoxylin, macroscopic cell colonies scored, and expressed as percent commitment where 0% commitment at time 0 corresponds to 426 colonies and 99% commitment at 24 h corresponds to four colonies. MM14 cells that have committed to a postmitotic phenotype do not proliferate, stain MHC positive as single differentiated cells, and thus are not scored as macroscopic colonies. Each point represents the average percent commitment from duplicate dishes.

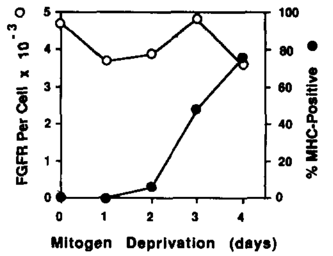

Figure 7. FGFR behavior and myosin accumulation during BC3H1-A1 cell differentiation. BC3H1-A1 cells were grown in DME plus 20% FCS for 24 h, rinsed three times with Puck's Saline G, and switched to DME plus 0.5% FCS for 4 d. On each day the FGFR number per cell was determined by equilibrium binding with 200 pM ^{125}I-aFGF in the presence and absence of 20 nM aFGF. The cells were processed for ^{125}I-aFGF binding as described in Materials and Methods. Parallel cultures were fixed, stained for MHC (Materials and Methods), and scored for the percent MHC-positive cells relative to the total cell number present. The data points represent mean values from triplicate determinations. This experiment was repeated twice with similar results.

Analysis of FGFR Behavior during BC3H1-A1 Muscle Cell Differentiation

To further investigate the relationship between FGFR loss and the postmitotic phenotype, FGFR behavior was studied during differentiation of the BC3H1-A1 smooth muscle cell line. Upon differentiation, BC3H1 cells undergo transcriptional induction of muscle-specific genes, but they do not fuse nor commit to a postmitotic phenotype (Schubert et al., 1974; Lathrop et al., 1985*a*, *b*). These cells also exhibit FGF-mediated repression of muscle cell differentiation (Lathrop et al., 1985*a*, *b*). BC3H1 cells thus offer a system for studying FGFR behavior in differentiating muscle cells that do not become postmitotic. When switched from medium containing 20% FCS to 0.5% FCS, BC3H1-A1 cells undergo a gradual increase in the number of MHC-positive cells. At 4 d, when 75% of the cells were MHC positive and not replicating, there was no change in the apparent FGFR number per cell (Fig. 7). Scatchard analysis of ^{125}I-aFGF equilibrium binding data to BC3H1-A1 cells confirmed this result, revealing no change in either the K_d for ^{125}I-aFGF binding or the FGFR number per cell (Table II).

Although the equilibrium binding data could not distin-

Table II. ^{125}I-aFGF Binding to BC3H1-A1 Cells

Proliferative state*	K_d‡	FGFR per cell
	pM	
Proliferating	15 ± 9	7,500 ± 2,000
Differentiated	14 ± 3	7,000 ± 1,500

* Cells were grown in DME and 20% FCS (proliferating) or 0.5% FCS (differentiated) for 4 d. Percent MHC positive cells were <1 and 75%, respectively.

‡ The data for K_d and FGFR number per cell were determined by Scatchard analysis of equilibrium ^{125}I-aFGF binding data. Mean and standard deviation from three independent experiments are reported.

guish whether the cell surface FGFR detected in differentiated BC3H1-A1 cultures was solely attributable to the 25% fraction of the cell population that failed to express MHC, this possibility was eliminated by single cell analysis of ^{125}I-aFGF binding (Fig. 1 *f*) showing uniform labeling over all cells in the culture. The failure of differentiated BC3H1-A1 cells to lose their FGFR thus appears to correlate with their failure to acquire a postmitotic phenotype.

Discussion

Terminal differentiation of skeletal muscle in culture is accompanied by a complete and permanent loss of cell surface FGF and EGF binding (Figs. 1 and 5). The loss of growth factor binding most likely represents a true loss of receptors from the cell surface, since for FGFR, the loss of binding capacity is not due to changes in FGF affinity (Fig. 3). Furthermore, since MM14 cells lose both EGFR and FGFR with a nearly identical time course (Figs. 4 and 5), it is also unlikely that FGFR loss is due to secretion of a soluble factor that competes for FGF binding. This conclusion is also supported by the fact that 16 h after FGF removal, FGFR- and EGFR-positive cells can be observed adjacent to mononucleated cells and myotubes that fail to bind to either growth factor (Lim and Hauschka, 1984*a*; Olwin, B. B., and S. D. Hauschka, data not shown). By 24 h FGFR appears to be lost not only from the cell surface but also appears to be eliminated from the entire cell because crude membrane preparations from lysed cells exhibit no specific ^{125}I-aFGF binding (data not shown).

FGFR loss is not peculiar to a single myogenic cell line since two independently derived skeletal muscle lines (MM14 and C2C12), which acquire a postmitotic phenotype during terminal differentiation, undergo a permanent loss of cell surface FGFR. Although both cell lines are repressed from terminal differentiation by FGF and lose their FGFR when mitogens are removed, only MM14 myoblasts are absolutely dependent upon added FGF for replication. Thus, FGFR loss during skeletal muscle development is not dependent on whether FGF is required for proliferation. That FGFR loss is associated with the postmitotic phenotype and not simply with the cessation of proliferation and the expression of muscle-specific genes is evident from the behavior of BC3H1-A1 cells. These cells become myosin positive in response to FGF and serum deprivation, but they do not lose their cell surface FGFR and do not become postmitotic.

While our data indicate that FGFR and EGFR are lost from the cell surface of differentiated skeletal muscle cells, not all "growth factor" receptors exhibit this behavior. Insulin receptors increase severalfold during L6 skeletal muscle differentiation while insulin-like growth factor (IGF) I and II receptors decrease severalfold, but do not disappear (Beguinot et al., 1985). Thus, while some growth factor receptors may undergo a coordinate decrease, others are clearly regulated by independent processes.

We previously observed that EGFR loss accompanies MM14 terminal differentiation and proposed that mitogen receptor loss may be a causal event for terminal skeletal muscle differentiation (Lim and Hauschka, 1984*a*). However, since MM14 cells are not responsive to EGF, it was unclear whether the disappearance of EGFR would be an accurate indication of FGFR behavior during MM14 differentiation. These previous observations have now been extended by demonstrating that the loss of EGFR and FGFR occur with nearly identical kinetics (Figs. 4 and 5). The transient rise in these cell surface receptors, an immediate consequence of growth factor removal, most likely results from a cessation of rapid, ligand-induced internalization. The decline in these receptors, which begins 12–16 h after FGF removal, is presumably due to the cessation of EGFR and FGFR biosynthesis many hours earlier, followed by the subsequent depletion of the intracellular and cell surface receptor pools via an intrinsic degradation process. The additional observation that MM14 cell surface EGFR expression is absolutely dependent on added FGF but not on EGF, and that removal of FGF in the continued presence of EGF results in loss of EGFR (unpublished data), suggests that FGFR and EGFR as well as other mitogen receptors could be developmentally regulated by a common mechanism during skeletal muscle differentiation. Since transforming growth factor-β (TGF-β) is also a repressor of myogenic differentiation (Florini et al., 1986; Massagué et al., 1986; Olson et al., 1986), our model predicts that a loss of TGF-β receptors is likely to occur during terminal differentiation of muscle cell lines such as C2C12 and L6 that are responsive to TGF-β; and indeed this has been recently reported for both lines, whereas BC3H1 cells, as in our study of FGFR loss, exhibited only a minimal decline in TGF-β receptors (Ewton et al., 1988).

Our results demonstrate that the decline in cell surface FGFR lags behind the loss of FGF growth responsiveness by at least 8 h (Fig. 6), and thus an immediate loss of cell surface FGFR per se is unlikely to be the causal event for MM14 commitment to a postmitotic phenotype. Furthermore, the cell surface FGFR level is actually two- to threefold higher in committed cells 12 h after FGF removal than in proliferating cultures. Even though FGF affinity for FGFR is unchanged during this period (Fig. 3; Table I), restoration of FGF does not induce proliferation. This apparent paradox could be explained by a model in which FGFR synthesis as well as that of other mitogen receptors is abruptly terminated when the skeletal muscle terminal differentiation process is initiated. Even though the cell surface FGFR level is elevated 12 h post-FGF withdrawal, the residual FGFR stores may be insufficient to provide an adequate proliferation signal when FGF is restored. In support of this hypothesis are the observations that quiescent cells require a sustained (6–8 h) exposure to mitogens before they reenter S phase (Carpenter and Cohen, 1976; Aharanov et al., 1978; Schechter et al., 1978), and that S phase entry of mitotically synchronized MM14 cells requires 6–8 h of continuous FGF exposure (Linkhart, T. A., and S. D. Hauschka, unpublished observations).

Alternatively, differentiating skeletal muscle cells may first acquire a postmitotic phenotype due to the blocking of one or more intracellular mitogenic signals. This possibility has been investigated in L6E9 rat cells in which cultures containing extensive myotubes still exhibited induction of c-*myc* mRNA in response to serum addition (Endo and Nadal-Ginard, 1986). If the c-*myc* induction did indeed occur in myotubes, and not in residual single cells that may have escaped elimination during a cytosine arabinoside treatment, then it is clear that at least some types of postmitotic muscle cells retain portions of a putative mitogenic signalling pathway.

The skeletal muscle postmitotic state has also been investigated with heterokaryon techniques (Clegg and Hauschka, 1987). In these studies postmitotic myocytes were fused with G_1 myoblasts and the heterokaryons were then exposed to FGF-containing medium plus [^{3}H]thymidine to determine whether either or both nuclei exhibited DNA synthesis. In heterokaryons containing ratios of even three G_1 nuclei to one postmitotic nucleus the post-mitotic phenotype is dominant; such cells exhibited no DNA synthesis and lost their cell surface EGF receptors. These results are thus consistent with either a receptor loss model for the postmitotic cells state or with a model in which postmitotic cells contain one or more *trans*-acting factors that block mitogen-mediated intracellular signals.

The in vivo behavior of muscle satellite cells represents an intriguing aspect of the mitogen receptor control model we have proposed for skeletal muscle development. These cells, which may remain quiescent and undifferentiated for years in adult muscle, reside beneath the basement lamina and in direct contact with the fully differentiated muscle fiber (Mauro, 1961; Schultz, 1976). Upon muscle injury the satellite cells are stimulated to undergo many rounds of replication, followed by fusion to form new muscle fibers (Snow, 1977; 1978). In vitro satellite cell growth (Bischoff, 1975; Konigsberg et al., 1975) can be stimulated by a factor from crushed skeletal muscle or by FGF, but the mitogenic effect of both factors also requires the simultaneous presence of serum (Bischoff, 1986*a*, *b*). To conform with the skeletal muscle growth factor receptor control model, during their in vivo developmental origin, satellite cells must retain their FGFR while neighboring muscle cells lose their FGFR, acquire a post-mitotic phenotype, and fuse into adjacent myotubes. In this model, the satellite cell FGFR must remain occupied by FGF thereby repressing the onset of terminal differentiation while the cell is simultaneously deprived of one or more other factors required for its continuous replication. In such an environment the satellite cell could then remain quiescent and undifferentiated as does the MM14 satellite cell line in the presence of a high FGF-low serum environment (Fig. 2; Clegg et al., 1987). Since FGF is present in adult and embryonic skeletal muscle (Kardami et al., 1985*b*; Seed et al., 1988), and since FGF may be localized in the extracellular matrix (Baird and Ling, 1987; Vlodavsky et al., 1987), the possibility of satellite cells being continually exposed to FGF in vivo seems plausible. Additional factors in serum, which appear to be required for satellite cell proliferation, would presumably be accessible only upon muscle fiber injury. In the presence of FGF the serum factors would then promote satellite cell proliferation and fiber regeneration.

The permanent loss of certain growth factor receptors, which we have reported in this and earlier studies (Lim and Hauschka, 1984*a*), could be an in vivo control process for maintaining the terminally differentiated state in skeletal muscle as well as a general regulatory mechanism for the sequential development of other tissues (e.g., neurons). By losing specific receptors at a particular developmental stage cells would be prevented from continued replication and from "reversing" their developmental pathways since they would then be unresponsive to the factors that control the growth and differentiation of their immediate precursors. Such a regulatory system has the advantage of permitting some cells to cease replicating and to express differentiated functions at an early developmental time while other cells of the same type remain proliferative and expand the tissue's total cell mass. If this model is pertinent to tissue development, critical mechanistic questions remain as to the nature of the microenvironment and/or cellular differences that are responsible for generating such temporal diversity in cell behavior, and how the expression of specific receptors is regulated.

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