Korean J. Zool., 32:163-175, 1989

Alteration in the Transferrin Receptor during the Chick Myoblast Fusion in Culture

Chang Ho Lee, Byung Je Yoo*, Young Ju Jeon, Chin Ha Chung, and Doo Bong Ha

*Dept. of Zoology, Seoul National University, Seoul 151-742; *Dept. of Biology, Taegu university, Taegu 705-030, Korea*

Transferrin (Tf) has been known to exert profound effect on the myoblast differentiation *in virto*. Therefore, the changes in the amount and affinity of the Tf receptor would accompany the myoblast differentiation. To investigate this possibility, we examined the alteration pattern in the level of the Tf receptor during the myoblast fusion.

The level of Tf receptor was assayed by measuring the bound ^{125}I-Tf onto the surface of cultured myoblasts, and it was known that the level of Tf receptor reached the maximum at about 12 hr before the initiation of the myoblast fusion and decreased as the differentiation proceeded, and that the affinity of Tf receptor to Tf was also decreased. In addition, various inhibitiors of the myoblast fusion also influenced the level of the Tf receptor.

Accroding to these results, it is postulated that the level of Tf receptor is highly regulated during the myoblast differentiation.

KEY WORDS:Transferrin receptor, Chick myoblast differentiation

Transferrin (Tf) is an iron-binding glycoprotein that has recently received considerable attention because of its profound effect on the proliferation and terminal differentiation of various cell types (Yeoh and Morgan, 1979). Concerning with the effects of Tf on the terminal differentiation, Ekblom *et al.* (1981) showed that Tf promoted the kidney cell differentiation and no other serum factor could replace it. They have suggested that Tf is a general regulator of embryonic growth and differentiation (Ekblom *et al.*, 1983), and argued that the acquisition of Tf responsiveness is a prerequisite event for the kidney cell differentiation. However, whether the acquisition of Tf responsiveness is related with the expression of Tf receptor is not revealed. On the other hand, the cultured chick myoblast, unlike kidney cells mentioned above, does not need the inductive events to respond to Tf (li *et al.*, 1982). Thus Tf could promote chick myoblast to proliferate and to differentiate without any inductive events. More recently, Hagiwara *et al.* (1987) have reported that the high concentration of Fe^{3+} in the basal medium could produce the myotrophic effects to the same degree as diferric Tf did. According to these results, the interaction between Tf and its receptor does not play a primary role such as a cell signalling in the myoblast differentiation. However, because Tf is the obligatory physiological iron donor and has a profound effect on the differentiation of myoblast *in vitro*, the regulation of expression of the Tf receptor is crucial events in the course of myoblast differentiation.

The Tf receptor is a major surface membrane protein of most rapidly proliferating cells (Aisen and Liskowsky, 1980), and thus it is appreciated as a specific surface marker for these cells (May and Cuartrecasas, 1985). The receptors are also highly expressed on the surface of various differentiating cells, e.g. PHA-stimulated human T

cells (Pauza *et al.*, 1984), differentiation-induced erythroleukemia cells (Hu *et al.*, 1977; Neckers *et al.*, 1986), and maturing chick reticulocytes (Schmidt *et al.*, 1986a). Interestingly the terminal differentiation is followed by the sharp reduction in the number of Tf receptors (Hu *et al.*, 1977; Pan *et al.*, 1983; Schmidt *et al.*, 1986a). Thus it could be thought that the Tf receptor perform a critical role in the initiation of terminal differentiation of various cell types (Iacopetta *et al.*, 1982).

In the muscle cell, the relationship among Tf, Tf receptor, and cellular differentiation has been also investigated to reveal the changes in the Tf receptor level during the myogenesis *in vitro*. It has also been previously shown that chick embryo extract is an absolute requirement for the myoblast fusion *in vitro*, and that the myotrophic activity of embryo extract is mostly due to the presence of Tf (Ii *et al.*, 1982). Hasegawa and Ozawa (1982) reported that, in spite of the myotrophic activity of Tf, the differentiated myotubes have more Tf receptors on their surface than the myoblasts. Sorokin *et al.* (1987) also found that the number of Tf receptors and the rate of endocytosis of Tf were maximum in the myotube stage. These results imply that Tf does not concern with the myoblast fusion *per se* but play a significant role as an iron donor during the myotube maturation.

The Tf receptor has been identified and characterized in a wide variety of cells *in vitro*. Hunt *et al.* (1984) and Wada *et al.* (1979) have characterized a 90 kD protein as the Tf receptor of cultured human T lymphocyte and human placenta. The gene for the Tf receptor has been also characterized and cloned by McClelland *et al.* (1984), and Schneider *et al.* (1983).

In the present experiment, the relationship between the Tf receptor and the myoblast differentiation was studied by measuring the change in the Tf receptor level on the cell surface. It was known that the number of Tf receptors and the affinity of the receptor for Tf reached the maximum just prior to the initiation of myoblast fusion and began to decrease drastically thereafter. In addition, the level of the Tf receptor was influenced by various fusion inhibitors with different mode according to the kind of inhibitors. Immunoprecipitation study revealed a 57 kD protein as the Tf receptor of chick myoblast.

Materials and Methods

Cell Culture

Embryonic myoblasts were obtained from breast muscle of 12-day chick embryo as described elsewhere (Ha *et al.*, 1983). Cells were plated at a concentration of 5×10^5 cells per ml of 811 medium (8 vol of minimum essential medium (MEM), 1 vol of horse serum, 1 vol of chick embryo extract, and 0.1 vol of antibiotic-antimycotic solution), and after 24 hr the medium was changed with 8102 medium (8.8 vol of MEM, 1 vol of horse serum, 0.2 vol of chick embryo extract, and 0.1 vol of antibiotic-antimycotic solution). At the same time, various fusion inhibitors were added to the medium.

Transferrin Purification and Antiserum Preparation

Tf was purified from 12-day chick embyo extract as described elsewhere (Yoo *et al.*, 1988) with some modification. The fraction of embryo extract precipitated in 55-70% ammonium sulfate was eluted with 0-100 mM linear salt concentration in DEAE-cellulose column that had been equilibrated with 10 mM Tris/HCl (pH 8.0). Tf was eluted at about 50 mM of salt concentration. The Tf fraction was heat-treated at 70°C for 10 min and followed by centrifugation at 15,000 x g for 30 min. The supernatant was collected and used as purified Tf. These Tf fraction revealed 80 kD single band with 99% purity on the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Horse Tf was purified from horse serum (Gibco) by the same method as described above. Tf was iron-saturated as described (Ii *et al.*, 1982), and radio-iodinated by the method of chrolamine T (Hunter and Greenwood, 1962) to give a specific activity of 1,000 cpm/fmol Tf. The antiserum against Tf was prepared by injecting 0.1 mg of the purified Tf emulsified with complete Freund's adjuvant. Two weeks later, the rabbit received three booster injections at weekly intervals of 0.1 mg of the Tf in incomplete adjuvants. Rabbit was then bled by heart puncture, and the IgG fraction was isolated by sodium sulfate fractionation (Johnstone and Thorpe, 1982).

Binding Assay of Transferrin

To assay the binding of Tf to its receptors on the cell surface, the cells were incubated in the ice-cold binding buffer (MEM buffered to pH 7.5 with 20 mM Hepes containing 2 mg/ml BSA, 150 μM $FeCl_3$, 10 mM $NaHCO_3$, and 10^{-7}M ^{125}I-labeled Tf) for 1 hr. Before the incubation, the cultured cells were thoroughly washed with warm MEM, incubated in MEM for 30 min, and again washed with the MEM buffered to pH 7.5 with 20 mM Hepes. $FeCl_3$ and $NaHCO_3$ were added into the binding buffer just prior to start of binding reaction. The non-specific binding was measured by carrying out the reaction in the presence of 2×10^{-5}M conalbumin.

The binding reaction was stopped by rinsing the cells 4 times with ice-cold MEM buffered with 20 mM Hepes at pH 7.5. The cells were then solubilized in 1 N NaOH and counted for their radioactivity with a γ-counter.

Immunocytochemical Staining of Cultured Muscle Cells

In order to observe the binding of Tf to its receptor on the cell surface, the cells were incubated in the culture medium (8102 medium) containing 2×10^{-7}M Tf for 15 min. The cells were then washed three times with phosphate buffered saline (PBS), fixed with 0.25% of glutaldehyde in PBS for 30 min, and washed three times with 2 mg/ml $NaBH_4$. Three hundred microliters of anti chick-Tf rabbit antiserum, 1:100 diluted with PBS containing 2 mg/ml BSA, was added to the fixed cell and incubated at room temperature with gentle agitation. After 3 hr the cells were washed five times with ice-cold PBS followed by a similar incubation with alkaline phosphatase linked anti rabbit-IgG goat antibody for 5 hr followed by 5 times rinses with PBS. The reaction for alkaline phosphatase was carried out using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates in 100 mM Tris/HCl (pH 9.5), 100 mM NaCl, 10 mM $MgCl_2$, and then stained cultures were microphotographed.

Immunoprecipitation of Transferrin Receptor from Cultured Myoblasts

In order to isolate and characterize the Tf receptor by immunoprecipitation method, the 24 hr cultured cells were first metabolically labeled with [^{35}S] methionine for 12 hr. Then Tf was added to the culture medium at a final concentration of 2×10^{-7}M and the cells were incubated for 15 min. The cells were then rinsed 3 times with a buffer consisting of 10 mM Hepes (pH 7.5), 120 mM NaCl, 5 mM KCl, and 1.5 mM $MgSO_4$, and cross-linked by 50 μM dithiobis (succinimidyl propionate) in the same buffer for 15 min at room temperature. The reaction was stopped by rinsing the cells with Tris buffer (10 mM Tris/HCl pH 7.5, 150 mM NaCl) twice, and the cells were incubated for 4 hr in the Tris buffer. After the incubation, the cells were solubilized by 1% Triton X-100 and by sonication. The solubilized extract was then microfuged, and rabbit anti chick-Tf antiserum that had been previously fractionated by $NaSO_4$, was added to the supernatant to give a final concentration of 500 μg/ml. After incubation at room temperature for 1 hr, goat anti rabbit-IgG antibody was added and allowed to stand overnight at 4°C. The cell extract was then microfuged for 15 min at room temperature, and the precipitate was solubilized with 2% SDS in 10 mM Tris/HCl pH 7.5. The cleavage of cross-link was carried out with 5% (v/v) β-mercaptoethanol followed by boiling to complete denaturation. The sample was then subjected to SDS-PAGE and autoradiographed.

Result

Binding Properties of Transferrin

The amount of Tf receptors on the cell surface was estimated by measuring the binding of ^{125}I-Tf to the cultured cells. The binding of Tf to the receptor is rapidly reduced when Tf is iodinated and stored for a long time. This reduction seems to be due to the dissociation of Fe^{3+} and HCO_3^- from iodinated Tf. Therefore, we assayed the binding of Tf to the receptor in the presence of high concentration of $FeCl_3$ and $NaHCO_3$ (higher than 100 μM and 3 mM, respectively). As shown in Fig.1, the binding of Tf was enhanced by the

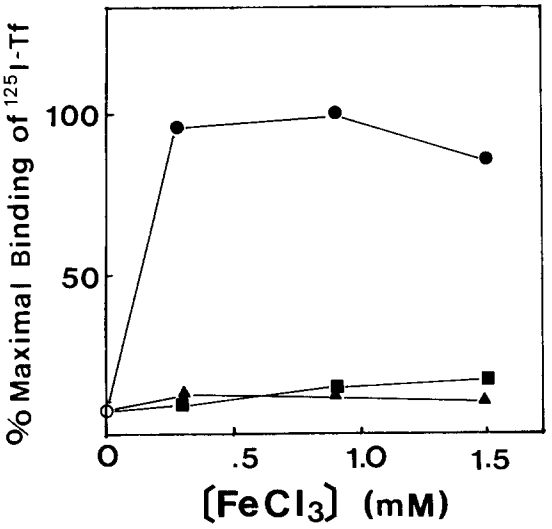

Fig. 1. Effects of the supplementation of $FeCl_3$ and $NaHCO_3$ on the binding of Tf to cultured myoblasts. The cultured cells were washed with MEM, and ^{125}I-Tf binding reaction was carried out in the MEM buffered at pH 7.5 with 20 mM Hepes at 4°C. Just prior to the initiation of Tf binding reaction, the supplements were added into the buffer to give the indicated concentration. After 1 hr incubation, the cells were washed with MEM, harvested and counted for their radioactivities (●, $FeCl_3$ plus $NaHCO_3$; ■, $FeCl_3$; ▲, $NaHCO_3$. The concentration of $NaHCO_3$ was always 10 times higher than that of $FeCl_3$).

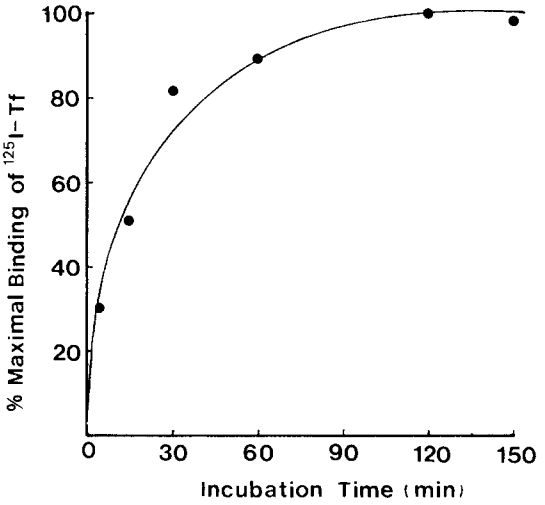

Fig. 2. Binding of ^{125}I-Tf to cultured myoblasts. Cells cultured for 24 hr in the 811 medium were rinsed with MEM and the binding reaction was carried out in the MEM containing 2 mg/ml BSA, 150 μM $FeCl_3$, 10 mM $NaHCO_3$, and 10^{-7}M ^{125}I-Tf at 4°C. The binding of Tf was ceased at the specified time.

presence of $FeCl_3$ and $NaHCO_3$ in the binding buffer. In this binding condition, the maxium binding of ^{125}I-Tf was achieved within 30 min after the binding reaction was started (Fig.2).

In order to illustrate the specificity of Tf binding, competitive binding assays were carried out with unlabeled Tf as inhibitor. The results obtained are shown in Fig.5. The binding of Tf to the cultured myoblast was saturated at a concentration range of Tf from 0.1 to 0.5 μM. The binding decreases when the cold Tf obtained from embryo extract or from horse serum was present in the binding buffer. As shown in Fig.5, horse serum Tf competes with chick Tf in binding to the surface of cultured myoblast cells, with somewhat lower affinity than chick Tf. Differences in the biological activity of two kinds of Tf were also examined, and the result is shown in Fig.4, which reveals that chick Tf is far effective in stimulating the myoblast fusion than horse Tf. Scatchard plot analysis of Tf binding re-

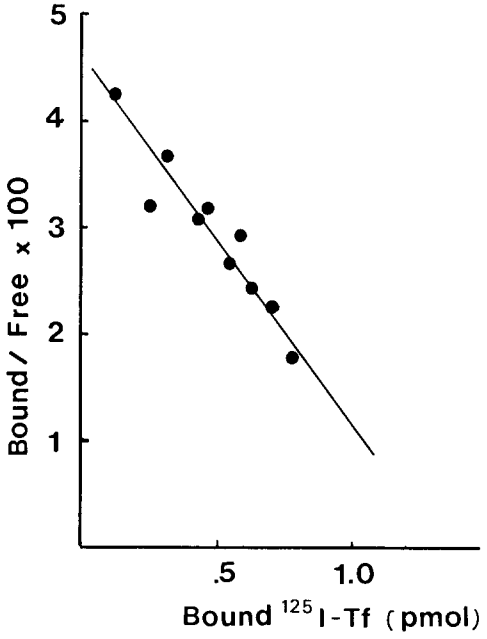

Fig. 3. The Scatchard plot analysis of ^{125}I-Tf binding to cultured myoblasts. Myoblasts were cultured in the 811 medium for 24hr, then the Tf receptors were assayed with different concentrations of ^{125}I-Tf for 60 min.

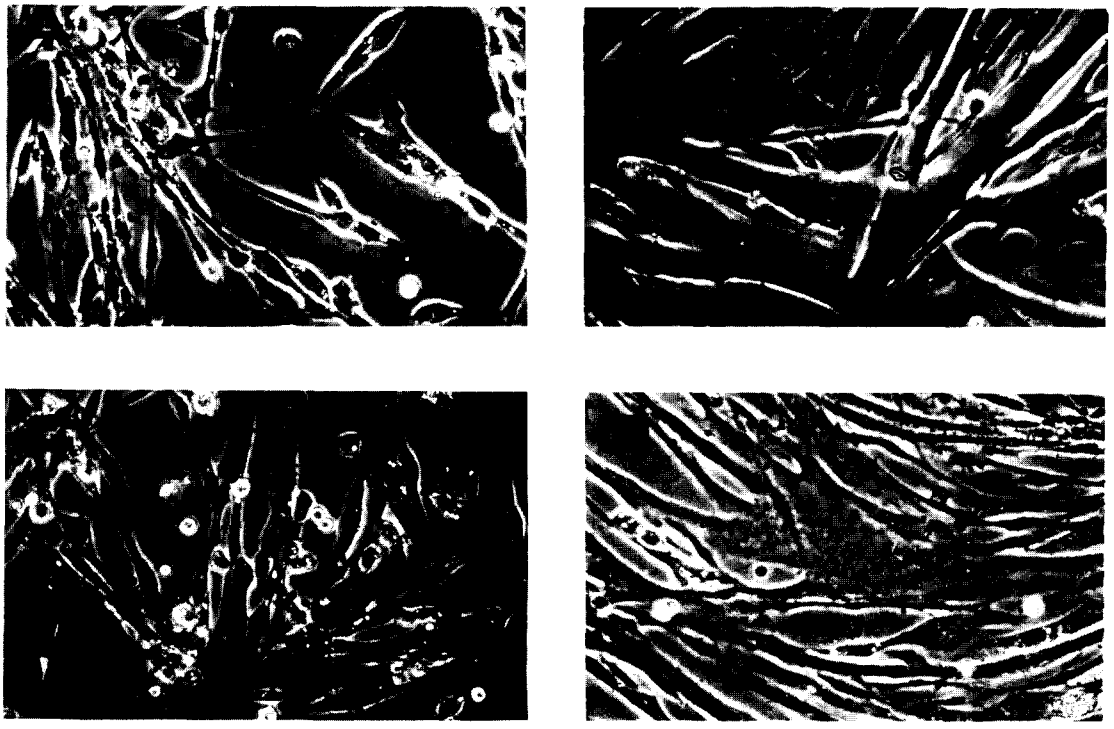

Fig. 4. Effects of chick Tf and horse Tf on the chick myoblast differentiation. After 24hr of the plating, the cells were rinsed with warm MEM for 3 times and subsequently cultured in the 910 medium (A), or in the 910 medium that was supplemented with 10^{-7}M chick Tf (B), 10^{-7}M horse Tf (C), and 2×10^{-6}M horse Tf (D). The cells were photographed after 48hr of the meidum change.

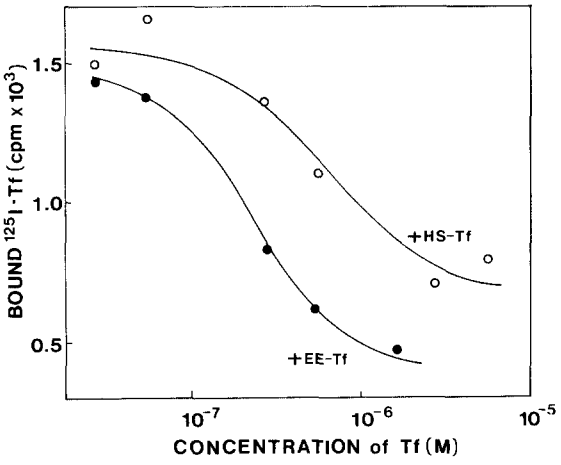

Fig. 5. Competitive binding of ^{125}I-chick Tf with unlabeled chick Tf (EE-Tf) and unlabeled horse Tf (HS-Tf). the Tf receptor was assayed using 10^{-9}M of ^{125}I-chick Tf in the presence of the indicated concentration.

vealed an apparent Kd (dissociation constant) $\cong$ 2.0×10^{-8}M and n (number of binding site) $\cong$ 5×10^5/nucleus of 24 hr cultured myoblast.

Changes in the Level of Transferrin Receptor During Myoblast Differentiation

In our culture condition, the myoblast fusion was initiated at about 48 hr and almost completed at about 60 hr after seeding. The synthesis of muscle specific proteins was dramatically increased during this period as previously reported (Ha *et al.*, 1983; Yoo *et al.*, 1988).

In the present experiment, the change in the level of Tf receptor during the myoblast differentiation was analyzed, and the result is given in Fig.6. Before the initiation of myoblast fusion, the level of the Tf receptor kept increasing until 36 hr of cultivation and then decreased dramatically with time.

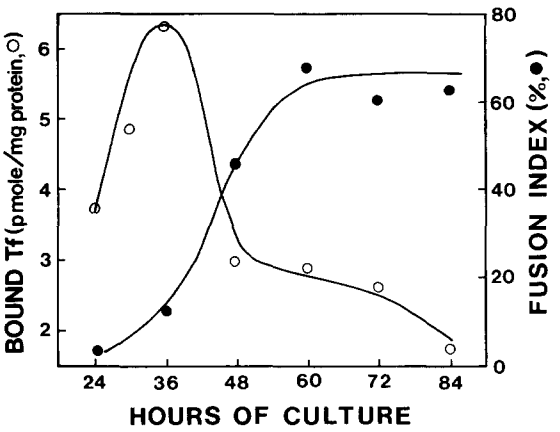

Fig. 6. Changes in the level of the Tf binding during myoblast differentiation. Myoblasts were cultured as described in "MATERIALS AND METHODS", and the Tf receptor assay was carried out at the indicated time.

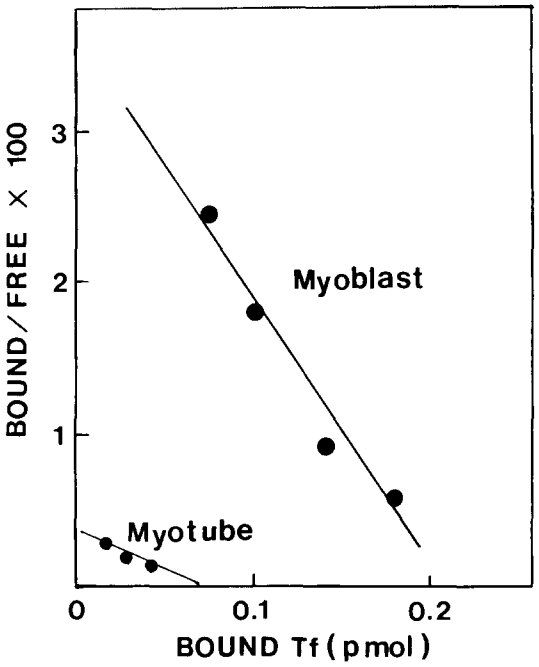

Fig. 7. Changes in the affinity of the Tf receptor for Tf during myoblast differentiation in culture. The Tf receptor was assayed with different concentrations of ^{125}I-Tf at 36 hr (myoblast), and at 72 hr (myotube) of cultivation. The dissociation constant was calculated as 1.9×10^{-8} for myoblast and 6.7×10^{-8} for myotube.

The time schedule of myoblast differentiation in the present experiment revealed some variation because of the use of the complex medium that contains chick embryo extract. The peak of the level of the Tf receptor, however, appeared always before the initiation of the myoblast fusion.

To examine whether the change in the level of Tf receptor was due to the change in the receptor number or due to the change in the receptor affinity for Tf, we compared the numbers and the affinity of the Tf receptor at myoblast stage and at myotube stage by Scatchard-plot analysis (Scatchard, 1949). As shown in Fig.7, the Tf receptor of myotubes revealed much lower level in their number and in their affinity for Tf than that of myoblasts. The apparent Kd was 2×10^{-8}M for myoblast stage and 7×10^{-8}M for myotube stage. Therefore, it was appeared that the change in the binding of Tf during myoblast differentiation is due to both changes in the number and in the affinity of the Tf receptor.

Effects of Fusion Inhibitors on the Level of Transferrin Receptor

Since the level of Tf receptor was highly associated with the stage of myoblast differentiation, it may be expected that fusion inhibitors would exert profound effects on the level of Tf receptor.

Addition to the 8102 medium of EGTA at a concentration of 2 mM inhibited the myoblast fusion but not inhibited the synthesis of muscle specific proteins. On the other hand, the addition of DMSO at a final concentration of 3% inhibited both events of myoblast differentiation (Fig.8). Diltiazem is an organic Ca^{2+} current inhibitor and has been reported to inhibit the expression of Tf receptor in the course of T cell activation (Neckers *et. al.*, 1986). It would be therefore interesting to examine whether diltiazem inhibit either the expression of Tf receptor of cultured myoblast or the myoblast differentiation. As revealed in Fig.9, the cells that were treated with DMSO or diltiazem maintained the constant level of the Tf receptor. EGTA-treated myoblasts, however, revealed less number of Tf receptors on their surface than the untreated control cells. Consequently the peak level of Tf receptor was not observed in the cells

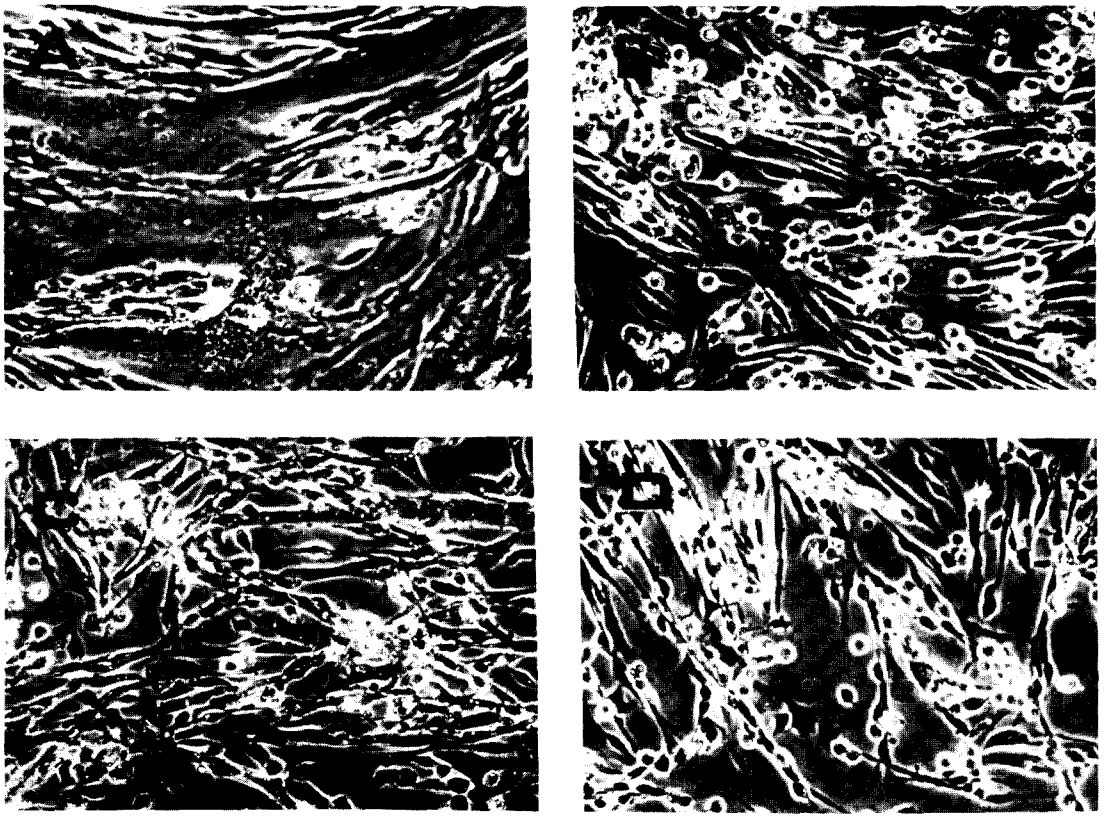

Fig. 8. Phase-contrast microphotographs of myoblasts that were cultured with various fusion inhibitors. At 24hr of cultivation, 811 medium was changed with 8102 medium (A), or the 8102 medium that was supplemented with 2 mM EGTA(B), 160 μM Diltiazem (C), and 3% DMSO (D). The cells were photographed at 72hr of cultivation.

that were treated with a fusion inhibitor. Thus the elevation of the level of Tf receptor seemed to be concerned with the myoblast fusion.

Immunocytochemical Staining of Cultured Muscle Cells

The binding of Tf to the receptor was analyzed by immunocytochemical staining of the cells. Fig.10 shows the microphotographs of immunocytochemical staining of cultured myoblasts. At 72 hr after seeding, most myoblasts have been fused to form thick myotubes (Fig.10). The location of Tf binding was observed mainly in the remaining unfused myoblasts and not in the myotubes.

However, when the cultures were treated with EGTA, the cells were not reactive to the Tf antibody, even though they were mononucleated myoblasts. On the other hand, the mononucleated cells were stained by the Tf antibody when the cells were fusion-inhibited by DMSO (Fig.10). These results were consistent with that of Fig.9. Interestingly, the cells that were treated with high concentration of embryo extract were reactive to the Tf antibody at 72 hr of cultivation (Fig.10).

Immunoprecipitation of Transferrin Receptor

The Tf receptor was isolated and characterized by immunoprecipitation method. The cultured myoblasts were first metabolically labeled with [^{35}S]-methionine, and subsequently solubilized in Triton X-100 followed by sonication, and the Tf receptor was immunoprecipitated using rabbit anti

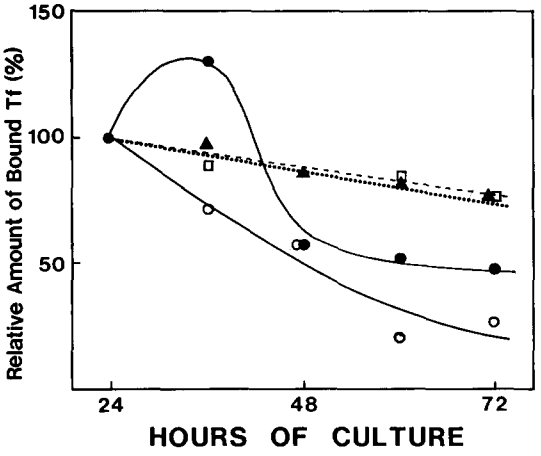

Fig. 9. Effects of various cell fusion inhibitors on the level of the Tf binding to cultured myoblast. The cultured cells were treated with various fusion inhibitors as described in the legend of Fig. 7, and the Tf receptor assay was carried out at the indicated time of cultivation. (●, 8102 control; ○, EGTA, ▲, diltiazem; □, DMSO).

chick-Tf antibody and goat anti rabbit-IgG antibody. Because Tf receptors were crosslinked with Tf before the cell solubilization, Tf receptor would coprecipitate with Tf.

Immunoprecipitant was solubilized with 2% SDS, subsequently reduced with β-mercaptoethanol, boiled to complete denaturation and finally subjected to SDS-polyacrylamide gel electrophoresis. Autoradiogram of the resultant gel revealed a 57 kD protein to be the Tf receptor of chick myoblast (Fig. 11).

Discussion

Transferrin (Tf) is a glycoprotein that transport iron to all tissue cells from the liver and the intestine. It is a single polypeptide chain, of molecular weight in the range 76kD-81kD, with two similar but not identical iron binding sites and sites for anion, i.e. probably carbonate ion (Aisen and Liskowsky, 1980). The binding and release of iron by Tf is significantly influenced by the presence of carbonate ion. Under physiological condition iron is tightly bound to Tf, but, in the absence of suitable anion this binding of iron does not occur at all (Bates and Schlabach, 1975). Because the iron-free apo-Tf has much lower affinity for its receptor than the iron-saturated holo-Tf, high concentrations of Fe^{3+} and HCO_3^-ion would facilitate the Tf binding to its receptor, and it was the case in the present experiment. As revealed in Fig. 1, high concentrations of Fe^{3+} and HCO_3^-in the binding buffer dramatically increased the binding of Tf to its receptor. Therefore we measured the Tf binding in the presence of 150 μM $FeCl_3$ and 10 mM $NaHCO_3$ throughout.

We examined whether horse Tf could bind to Tf receptor of chick myoblast. While Tf is generally known as a species-specific molecule, it was known in the present experiment that horse Tf bind to Tf receptor of chick myoblasts and, moreover, it induced chick myoblast to differentiate although at much higher concentrations (Figs. 4 and 5). It was apparent that, as revealed in Figs. 4 and 5, the affinity of horse Tf for chick myoblast surface receptor is far lower than that of chick embryonic Tf. These results provide a probable interpretation for the previous report that free iron could support the myogenesis to the same degree with Tf (Hagiwara *et al.*, 1982). Free iron ion would be carried into the chick myoblast by horse Tf in the medium and through the Tf receptor on the surface of cultured chick myoblast. Thus the indispensability of Tf in the chick myogenesis is not excluded. To elucidate more clearly the requirement of Tf and its receptor in the myogenesis, it would be necessary to use a serum-free defined medium or the specific ionophore bypassing the Tf for delivering iron into the cell.

Many reports suggested that the interaction between Tf and its receptor plays a crucial role in the cellular metabolism related to growth and differentiation. For example, a marked increase of Tf binding sites occurred in antigen-stimulated lymphocytes (Pauza *et al.*, 1984) and differentiation-induced erythroleukemic cells (Yeh *et al.*, 1982; Hu *et al.*, 1977). and erythroblasts (Schmidt *et al.*, 1986b). In the present study, the number of Tf binding sites on the surface of chick myoblasts were found to increase after 811 medium was

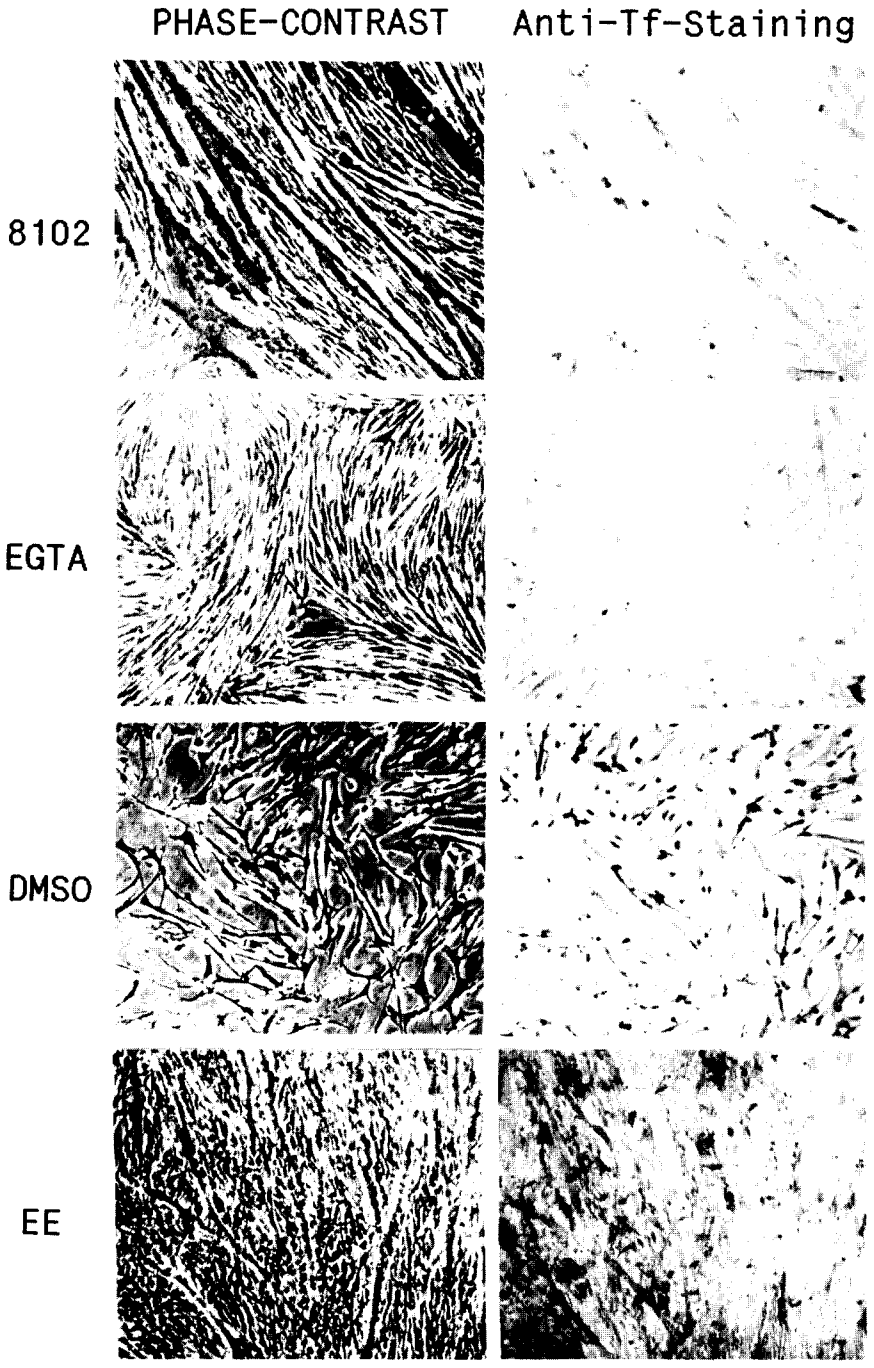

Fig. 10. Immunocytochemical staining of the cultured myoblast. At 72 hr of cultivation, the cells were incubated with 2×10^{-7}M of Tf for 15 min, fixed with 0.25% of glutaldehyde in PBS for 30 min, and washed with 2 mg/ml $NaBH_4$ chick-Tf antiserum that was diluted with $\times 10$ vol of PBS containing 2 mg/ml BSA for 3hr with gentle agitation followed by the 4 times rinsing with PBS. Then the cells were treated with alkaline phosphatase conjugated goat anti-rabbit IgG antibody with similar incubation. After rinsing with PBS for 5 times, reaction for alkaline phosphatase was carried out for 30 min as described in "Materials and Methods".

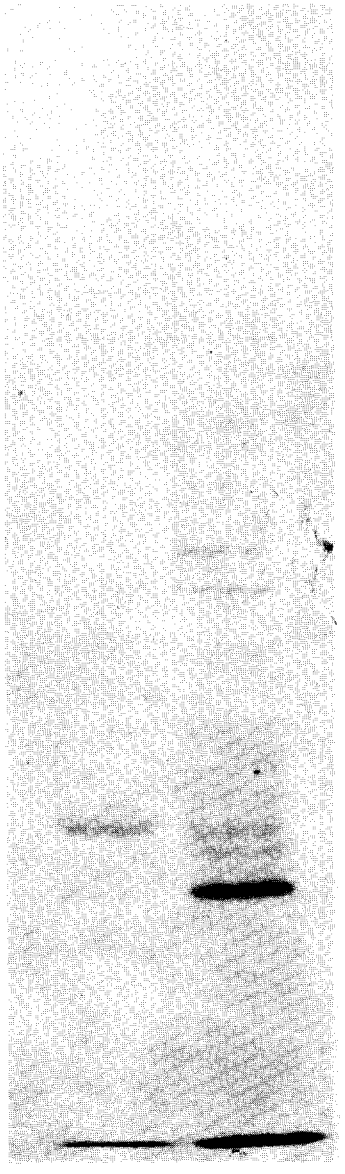

Fig. 11. Autoradiogram of the immunoprecipitated Tf receptor using anti chick-Tf antibody. Tf was added into the culture medium, and crosslinked by dithiobis (succinimidyl propionate) with the Tf receptor, followed by rinsing with Tris buffer. Then the myoblasts were harvested in 1% Triton X-100. To complete solubilization the cells were sonicated briefly, then microcentrifuged for 15 min. Rabbit anti chick-Tf antibody was added into the collected supernatant followed by the incubation for 1 hr at room temperature. Then the goat anti rabbit-IgG antibody was added again into the sample followed by the overnight incubation at 4°C. After the incubation, the sample was microcentrifuged for 15 min, and the precipitate was rinsed with 1% Triton X-100 again. The resultant precipitate was solubilized with 1% SDS, and was subjected to SDS-PAGE on 8% acrylamide gel. The resultant gel was fluorogaphed and exposed to X-ray film for 1 week at-70°C. (A; precipitate, B; supernatant)

changed to 8102 medium, with the maximum level at 36 hr of cultivation. The number was then abruptly reduced with the time (Fig. 6). In previous papers (Sorokin *et al.*, 1987; Hasegawa and Ozawa, 1982), it was reported that differentiated myotubes showed higher level of Tf receptor and the rate of iron uptake over undifferentiated myoblasts. The discrepancy between our result (Fig. 6) and the above reports would be partly due to the difference in the Tf binding condition. Fig. 6 reveals similar results to that obtained from the erythroleukemic cell system (Yeoh *et al.*, 1982; Pan *et al.*, 1983; Schmidt *et al.*, 1986a and 1986b), since the Tf binding sites of myoblasts were reduced to low level as the terminal differentiation proceeds. Since the level of Tf receptor in the present experiment is expressed as bound Tf per unit amount of protein, it could be thought that the level of Tf receptor would decrease as the total amount of protein increases with time. However, the total amount of protein was not significantly changed during the period of 36hr-48hr of cultivation. During this period, the amount of bound Tf per culture plate was dramatically reduced.

Intriguing result is shown in Fig. 7 which reveals that the affinity of Tf receptor was also reduced during the myogenesis in culture. The changes in the affinity of Tf receptor might be concerned with the chemical modification, such as phosphorylation (May *et al.*, 1986) or covalent conjugation with lipid (Omary and Trowbridge, 1981). Because the concentration of Tf in the serum is much higher level than the dissociation constant calculated from the Scatchard plot, the change in the affinity of Tf receptor would have no biological significance in the regulation of myogenesis *in vivo*. However, in developing chick embryo and in the rapidly proliferating tissue, the cells would be subject to restriction in interacting with Tf. Thus, to elucidate the biological significance of al-

teration in the affinity of Tf receptor, the availibility of Tf in chick embryonic muscle tissue should be determined first.

Various growth factors and chemicals were used to reveal the precise relationship between Tf and differentiation (Besancon *et al.*, 1985; Neckers *et al.*, 1986; Neckers and Cossman, 1983; Yeoh and Morgan, 1979). Many studies suggested close correlationship between them. For example, the growth factor and the chemicals which inhibit the expression of Tf receptor gene were also inhibitory for biological response to Tf (Besancone *et al.*, 1985; Neckers *et al.*, 1983 and 1986). Therefore, we have examined whether the chemicals inhibiting the myoblast fusion affect the Tf receptor level of myoblasts. EGTA, previously reported as a myoblast cell fusion inhibitor (Endo and Nadal-Ginard, 1987), drastically reduced the Tf receptor level, and inhibited the myoblast fusion at the same concentration (Figs. 8 and 9). Dimethylsulfoxide and diltiazem retained the Tf receptor at constant level that is revealed at 24 hr cultured myoblasts (Fig. 9). Previous report (Endo and Nadal-Ginard, 1987) has shown that EGTA inhibits chick myoblast fusion but not the expression of muscle specific proteins and the proliferation of myoblast. Since iron is an essential element for cells, it may seem to be surprising at first that the reduction of Tf receptor level does not affect cell proliferation. EGTA, however, does not thoroughly diminish the Tf receptor level, and the supply of iron into the cell was not blocked completely by the treatment of EGTA. In addition, this result may imply that the Tf receptor of chick myoblast might not exist in an identical form. Immunocytochemical staining revealed again that the Tf receptor level was influenced by inhibitors of cell fusion. In addition, the fusion-blocked myoblast by embryo extract retained relatively high level of Tf binding sites until 72 hr of cultivation.

With regard to the molecular weight of Tf receptor, Hunt *et al.* (1984) have reported as a Tf receptor in cultured human T lymphocyte, and Schmidt *et al.* (1986 and 1986a) claimed it as 90-95 kD in chick erythrocytes. However, Markelonis *et al.* (1985) have suggested a 57 kD protein in cultured chick nerve cells. Pauza *et al.* (1984) reported a translational product of Tf receptor mRNA as a 57 kD protein in antigen-stimulated human T lymphocytes using the Tf affinity column. Similar *in vitro* experiments (Schneider *et al.*, 1983) identified a 78kD translational product using either human placental RNA or RNA from the human lymphoblastoid HSB-7. In the present experiment, the immunoprecipitation of [^{35}S]-labeled cell homogenate gave a 57 kD protein as Tf receptor of chick myoblast (Fig. 11). It is interesting to infer that the different kinds of Tf receptor participate in the cell proliferation and in cell differentiation, since the Tf receptor that was identified in the rapidly proliferating cells revealed different molecular weight from that identified in the differentiating cells.

References

Aisen, P., and I. Liskowsky, 1980. Iron transport and storage proteins. Ann. Rev. Biochem. **49**:357-393.

Bates, G. W., and M. R. Schlabach, 1975. The nonspecific binding of transferrin in the absence of synergistic anions. *J. Biol. Chem.* **250**:2177-2181.

Besancon, F., M-F. Bourgeade, and U. Testa, 1985. Inhibition of transferrin receptor expression by interferon-α in human lymphoblastoid cells and mitogen-induced lymphocytes. *J. Biol. Chem.* **260**:13074-13080.

Ekblocm, P., E. Lehtonen, L. Saxén, and R. Timple, 1981. Shift in collagen type as an early response to induction of the metanephric mesenchyme. *J. Cell Biol.* **89**:276-283.

Ekblom, P., I. Thesleff, L. Saxén, A. Miettinen, and R. Timple, 1983. Transferrin as a fetal growth factor: Acquisition of responsiveness related to embryonic induction. *Proc. Natl. Acad. Sci. USA.* **80**:2651-2655.

Endo, T., and B. Nadal-Ginard, 1987. Three types of muscle-specific gene expression in fusion-blocked rat skeletal muscle cells: Translational control in EGTA-treated cells. *Cell* **49**:515-526.

Ha, D. B., B. J. Yoo, J. K. Sonn, H. S. Kang, and Y. S. Lee, 1983. Synthesis of muscle-specific proteins during the differentiation of chick embryonic muscle cells in culture. *Korean J. Zool.* **26**:1-17.

Hagiwara, Y., K. Saito, S. Atsumi, and E. Ozawa, 1987. Iron supports myogenic cell differentiation to the same degree as does iron-bound transferrin. *Dev. Biol.* **120**:236-244.

Hasegawa, T., and E. Ozawa, 1982. Transferrin receptor on chick fibroblast cell surface and the binding affinity in relevance to the growth promoting activity of transferrin. *Develop. Growth Differ.* **24**:581-587.

Hu, H. Y., Y. J. Gardner, P. Aisen, and A. I. Skoulich,

1977. Inducibility of transferrin receptors on Friend erythroleukemic cells. *Science* **197**:559-561.

Hunt, R. C., R. Ruffin, and Y-S. Yang, 1984. Alterations in the transferrin receptor of human erythroleukemic cells after induction of hemoglobin synthesis. *J. Biol. Chem.* **259**:9944-9952.

Hunter, W. M., and F. C. Greenwood, 1962. Preparation of Iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.)* **194**:495-496.

Iacopetta, B. J., E. H. Morgan, and G. C. T. Yeoh, 1982. Transferrin receptors and iron uptake during erythroid cell development. Biochim. *Biophys. Acta* **687**:204-210.

Ii, I. I. Kimura, and E. Ozawa, 1982. A myotrophic protein from chick embryo extract: Its purification, identity to transferrin, and indispensability for avian myogenesis. *Dev. Biol.* **94**:366-377.

Johnstone, A., and R. Thrope, 1982. Isolation of serum from blood. In: Immunochemistry in Practice, Blackwell Scientific publication, Melbourne, Australia, pp. 27-45.

Markelonis, G. J., T. H. Oh, L. P. Park, C. Y. Cha, C. A. Sofia, J. W. Kim, and P. Azari, 1985. Synthesis of the transferrin receptor by cultures of embryonic chicken spinal neurons. *J. Cell Biol.* **100**:8-17.

May, Jr. W. S., and P. Cuartrasas, 1985. Transferrin recptor: Its biological significance. *J. Mem. Biol.* **88**:205-215.

May, W. S., E. G. Lapentina, and P. Cuartrecasas, 1986. Intracellular activitation of protein kinase C and regulation of the surface transferrin receptor by diacylglycerol is a spontaneously reversible process that is associated with rapid formation of phosphatidic acid. *Proc. Natl. Acad. Sci. USA.* **83**:1281-1284.

McClelland, A., L. C. Kuhn, R. H. Ruddle, 1984. The human transferrin receptor gene: genomic organization, and the complete primary structure of the receptor deduced from a cDNA sequence. *Cell* **39**:267-274.

Neckers, L. M., S. Bauer, R. C. McGlennen, J. B. Trepel, K. Rao, and W. C. Greene, 1986. Diltiazem inhibits transferrin receptor expression and causes G1 arrest in normal and neoplastic T cells. *Mol. and Cell Biol.* **6**:4244-4250.

Neckers, L., and J. Cossman, 1983. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. *Proc. Natl. Acad. Sci.* USA. **80**:3494-3498.

Omary, M. B., and I. S. Trowbridge, 1981. Covalent binding of fatty acid to the transferrin receptor in cultured human cells. *J. Biol. Chem.* **256**:4715-4718.

Pan, B. T., R. Blostein, and R. M. Johnstone, 1983. Loss of the transferrin receptor during maturation of sheep reticulocytes *in vitro*. *Biochem. J.* **210**:37-47.

Pauza, C. D., J. D. Bleil, and E. S. Lennox, 1984. The contol of transferrin receptor synthesis in mitogen--stimulated human lymphocytes. *Exp. Cell Res.* **154**:510-520.

Scatchard, G., 1949. The attraction of proteins for small molecules and ions. *Ann. NY. Acad. Sci.* **51**:660-672.

Schmidt, J. A., J. Marshall, and M. J. Hayman, 1985. Identification and characterization of the chicken transferrin receptor. *Biochem. J.* **232**:735-741.

Schmidt, J. A., J. Marshall, M. J. Hayman, and H. Beug, 1986a. Primitive series embryonic chick erythrocytes express the transferrin receptor. Exp. Cell Res. **164**:71-78.

Schmidt, J., J. Marshall, M. J. Hayman, P. Ponka, and H. Beug, 1986b. Control of erythroid differentiation: Possible role of the transferrin cycle. *Cell 46:41*-51.

Schneider, C., M. J. Owen, D. Banville, and J. G. Williams, 1984. Primary structure of human transferrin receptor deduced from the mRNA sequence. *Nature (Lond.) 311:675*-678.

Sorokin, L. M., E. H. Morgan, and G. C. T. Yeoh, 1987. Transferrin receptor numbers and transferrin and iron uptake in cultured chick muscle cells at different stages of development. *J. Cell. Physiol.* **131**:342-353.

Wada, H. G., P. E. Hass, and H. H. Sussman, 1979. Transferrin receptor in human placental brush border membranes: Studies on the binding of transferrin to placental membrane vesicles and the identification of a placental brush border glycoprotein with high affinity for transferrin. *J. Biol. Chem.* **254**:12629-12635.

Yeh, C-J. G., M. Papamichael, and M. P. Faulk, 1982. Loss of transferrin receptors following induced differentiation of HL-60 promyelocytic leukemia cells. 1982. *Exp. Cell Res.* **138**:429-433.

Yeoh, G. C. T., and E. H. Morgan, 1979. Dimethyl sulfoxide induction of transferrin receptors on Friend erythroleukemia cells. *Cell Differ.* **8**:331-343.

Yoo, B. J., C. H. Lee, K. B. Kwak, C. H. Chung, and D. B. Ha, 1988. The presence in embryo extract of a myotrophic protein that affects proliferation and fusion of chick embryonic myoblasts in culture. *Korean J. Zool.* **31**:207-217.