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Changes in the Level of Transferrin Receptor during the Differentiation of Chick Brain Neuroblasts

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Insulin and transferrin (Tf) were found to be essential for survival and differentiation of brain neuroblasts obtained from chick embryo. This requirement, however, is changed from insulin to Tf upon neuronal development of the embryo, and this phenomenon is due to the changes in the levels of corresponding receptors. Using cultured neuroblasts, the level of Tf receptor is also found to increase while that of insulin receptor falls dramatically during the course of the cell differentiation. These results suggest that the development-specific changes in the levels of insulin and Tf receptors in embryo can be reproduced in the culture system during the differentiation period. Because the culture system used was a defined medium and contained no other macromolecules than insulin and Tf, it appears possible that insulin and Tf may act as signalling molecules in the control of neuronal development.

KEY WORDS: Transferrin, Insulin, Neuroblast, Differentiation

When dissociated neuroblasts are cultured under a proper condition, they stop dividing and undergo neuronal differentiation that is manifested by extention of long and branched neurites. Together with these morphological changes, synthesis of nerve-specific proteins related with neural function occurs; for example, catechol-o-methyltransferase (Wilson *et al.*, 1972), receptors for tetanus toxin (Mirsky *et al.*, 1978), and neurofilamentous proteins (Tapscott *et al.*, 1981). This biochemical differentiation, however, is not generalized because of their heterogeneity among cell types and plasticity that are susceptible to environmental changes (Black *et al.*, 1984). Therefore, the morphological change has still been generally used as the marker for differentiation of neuroblasts in culture.

Growth of neuron in culture requires the presence of serum in medium (Sensenbrenner *et al.*, 1980; Raizada 1983). The use of serum, however, has hampered the precise analysis of specific serum component(s) that regulates the neuronal development because of its complexed composition. A number of defined media have therefore been developed, and these media typically contain insulin and transferrin (Tf) in place of serum (Muller *et al.*, 1984; Aizenman *et al.*, 1986). In addition, it has been reported that insulin concentration in brain is 10- to 100-fold higher than that in plasma and the high level of plasma proteins, including Tf, are detected in the human and rat fetal cerebrospinal fluid (Jefferies *et al.*, 1984). Furthermore, brain neurons have been found to change their requirement from insulin to Tf for their survival and differentiation (Aizenman *et al.*, 1986). Thus, it appears likely that insulin and Tf play a critical role(s) in neuronal development.

As an attempt to elucidate the role of insulin

and Tf in the differentiation of cultured neuroblasts, we measured the changes in the receptor levels of insulin and Tf during the differentiation of cultured neuroblasts from embryonic brain.

Materials and Methods

Materials

All materials needed for neuroblast culture were obtained from Gibco. $Na^{125}I$ (100 mCi/ml) were purchased from Amersham. All other reagents were obtained from Sigma.

Tf was purified as described by Yoo *et al.*, (1988) and the purified Tf was then saturated with Fe^{3+} by following the method of li *et al.*, (1982). Radioiodination of Tf and insulin was carried out using chloramine T (Hunter and Greenwood, 1962). The specific activity of the labeled Tf was 3×10^7 cpm/μg, and that of insulin was 2.2×10^7 cpm/μg.

Neuroblast Culture

The cell cultures were performed as described by Aizenman *et al.* (1986). Briefly, telencephalons were dissected out form brains obtained from 6-, 7-, or 9-day-old chick embryo. They were minced with a forceps and passed through a 200 μm stainless filter. The dissociated cells were then collected by centrifugation at 700 $\times$ g for 6 min, and resuspended in the 1 : 1 mixture of Dulbecco's modified Eagle's medium (DME) and Ham's F12 medium (DME/F12 medium). When needed, insulin and/or Tf were supplemented to the medium. The cells were passed through 2 layers of lenz paper, and plated at a density of 5×10^5 cells/ml on 35 mm culture dishes that had been precoated with 4 μg/ml of poly-L-lysine (Yavin, 1974). The cultures were maintained at 37°C in 5% CO_2/95% humidified air. No refeeding was done during the entire culture period.

Receptor Assay

To assay the binding of Tf to its receptors on the cell surface, cultured neuroblasts were washed 3 times with warm MEM/Hepes (MEM buffered with 20 mM Hepes to pH 7.5) and subsequently incubated in a CO_2 incubator for 30 min. After the incubation, the cells were subjected to the binding reaction by washing with ice-cold MEM/Hepes and then incubation for 1 hr in the ice-cold MEM/Hepes containing 2 mg/ml BSA, 150 μM $FeCl_3$, 10 mM $NaHCO_3$ and 10^{-7} M ^{125}I-labeled Tf. The binding reaction was stopped by rinsing the cells 4 times with ice-cold MEM/Hepes. The cells were then solubilized in 1 N NaOH and counted for their radioactivity using a scintillation counter. The binding of ^{125}I-labeled insulin to its receptor was also assayed as above but using MEM/Hepes containing 2 mg/ml BSA and 2.5×10^{-8} M ^{125}I-insulin for 1 hr. Non-specific binding of Tf or insulin was determined using the same incubation mixture but in the presence of 1,000-fold excess of unlabeled Fe^{3+}-saturated Tf or cold insulin.

Microscopy

The cultured neuroblasts were washed with phosphate buffered saline (PBS) and fixed for 10 min in a mixture of 95% methanol, formaldehyde, and acetic acid (20 : 2 : 1 by volume). After rinsing with deionized water, the cells were observed under a phase-contrast microscope and those bearing neurites longer than 2-fold cell diameter were counted as a neurite-bearing cell. The percentage of the neurite-bearing cells was used as a differentiation index of neuroblasts in culture.

Results

Effect of Tf on Neuroblast Differentiation

To examine the effect of insulin and Tf on the survival and differentiation of cultured neuroblasts, the cells were obtained from 6- and 7-day-old embryonic brains and cultured in DME/F12 medium in the presence and absence of the proteins. For the cells from 6-day-old brain, supplement of a single hormone, insulin, was sufficient for bringing neurite outgrowth, a typical of neuronal differentiation (Fig. 1A). Treatment of Tf alone, however, could not support the cells to plate effectively or to extent their neurites properly (Fig. 1B). By contrast, in the culture from 7-day-old brain, a significant portion of the cells could not survive and extend neurites when insulin alone was treated (Fig. 1C). However, treatment of Tf into the medium could enhance the cells' ability to differentiate (Fig. 1D). These results

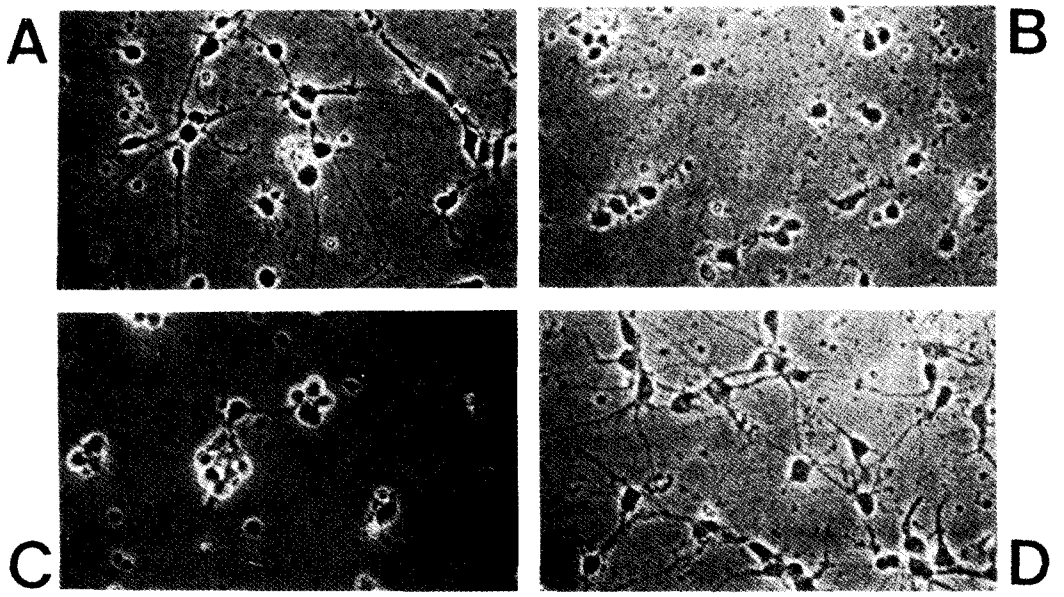

Fig. 1. Effects of insulin and Tf on neuroblast differentiation. Brain neuroblasts were obtained from 6- (A and B) and 7-day-old (C and D) chick embryo and cultured in DME/F12 medium supplemented with the followings: A and C, 10 μg/ml of insulin; B, 10 μg/ml. of Tf; D, 10 μg/ml of insulin plus 10 μg/ml of Tf. The cells were observed under a phase-contrast microscope at 72 hr of the culture.

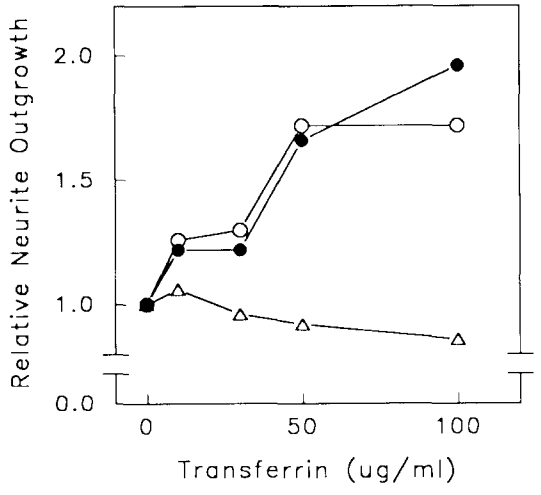

Fig. 2. Effect of increasing concentrations of Tf on neuroblast differentiation. The cells were obtained from 6-, 7- and 9-day-old chick embryo and cultured as in Fig. 1. but for 40 hr in the presence of 10 μg/ml of insulin and increasing amounts of Tf. Relative neurite outgrowth was expressed as the ratio of the number of neurite-bearing cells seen in the presence of Tf to those in its absence.

clearly suggest that the response to Tf varies by the age of embryo, from which the neuroblasts were obtained.

To examine further the development-dependent neurotrophic effect of Tf, neuroblasts were obtained from 6-, 7- and 9-day-old embryonic brains and cultured in the presence of increasing concentration of Tf. As shown in Fig. 2, Tf alone can support the suvival and differentiation of the cells from 7- and 9-day-old brain but not from 6-day-old brain. Thus, it appears quite clear that the neurotrophic effect of Tf is development-dependent. These results also suggest that the requirement for neuronal development changes from insulin to Tf in accord with the suggestion by Aizenman and coworkers (1986).

Changes in the Levels of Tf and Insulin Receptors

To examine if the changes in the Tf effect is due to the alterations in the level of Tf receptors during the developmental period, the brain neuroblasts were obtained from 6-, 7-, and 9-day-old chick embryos and cultured for 40 hr in DME/

F12 medium containing 10 μg/ml of insulin and 100 μg/ml of Tf. Tf receptors in the cells were then assayed by their ability to bind with ^{125}I-labeled Tf. Fig. 3 shows that the level of the Tf-receptor was about 2-fold higher in neuroblasts from 7- and 9-day-old brain than in those from 6-day-old brain. Thus, it is likely that the development-specific changes in Tf effect is mediated by the alterations in the level of Tf receptors.

We then tested if the increase in Tf level may also occur in differentiating neuroblasts in culture. Brain neuroblasts obtained from 6-day-old chick embryo were cultured in the presence of insulin and Tf, and Tf and insulin receptors were assayed at various time of the cultivation. As shown in Fig. 4, the level of insulin specific receptors gradually decreased to depletion by 4 days of culture while that of the Tf receptor increased particularly by the end of culture period. This inverse relationship of the receptor levels clearly reflects the changes in the requirement for neuronal development from insulin to Tf.

Discussion

Insulin and Tf have widely been used as essential supplements for a variety of cultured neuronal cells in the defined medium. In spite of their profound effects on neuronal survival and differentiation, they received little attention on the regulation of neuroblast differentiation partly because they also exert trophic effects on non-neuronal cell types. Using the pure brain neuroblast from chick embryo and a defined medium, the present studies demonstrated that both insulin and Tf are essential components for the cells' survival and differentiation and these requriements are changed from insulin to Tf during the neuronal development (Fig. 1) in accord with the earlier report by Aizenman and coworkers (1986). These results, however, were further potentiated by our additional findings that the level of Tf receptor increases in brain neuroblasts from developing embryo (Fig. 2). Thus, it seems clear that the development-specific effect of Tf is resulted from the increase in the level of Tf receptor during embryonic development.

Of particular interest was the finding that, during differentiation of the cultured neuroblasts, the level of insulin receptor undergoes a dramatic decrease while Tf receptor level rises. Thus, it appears that the culture system exerting the neuroblast differentiation resembles the neuronal development in embryonic brain. It is noteworthy,

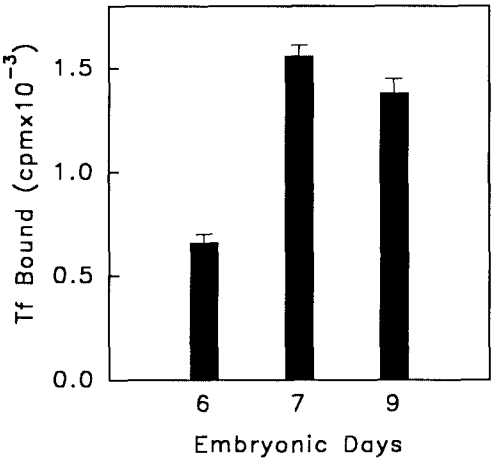

Fig. 3. Comparison of the Tf receptor level in cultured neuroblasts obtained from 6-, 7- and 9-day embryonic brain. Brain neuroblasts were obtained from variously aged chick embryo and cultured in DME/F12 medium supplemented with 10 μg/ml of insulin and 100 μg/ml of Tf. At 40 hr after the culture, the cells were assayed for their Tf receptor as described in Materials and Methods.

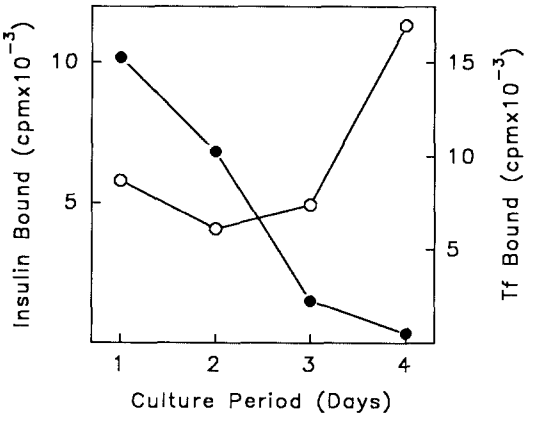

Fig. 4. Change in the levels of Tf and insulin receptors during the differentiation of cultured neuroblasts. Neuroblasts were obtained from 6-day chick embryonic brain and cultured in DME/F12 medium containing 10 μg/ml of insulin and 100 μg/ml of Tf. At the indicated time of the culture, the cells were assayed for their insulin (●) and Tf receptors (○) as in Materials and Methods.

however, there is no source for the cultured neuroblasts to obtain any signal or information from their environment that control the cell differentiation, because the defined medium used in the present studies contained no other macromolecules than insulin and Tf. Thus, the differentiation process that is manifested by the neuroblast culture is likely to be pre-scheduled phenomenon. In addition, it appears possible that insulin and Tf may act as signalling molecules in the control of neuronal development.

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계배 신경아세포의 분화에 따른 Transferrin 수용체의 변화

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Insulin과 transferrin(Tf)은 계배 신경아세포의 생존과 분화에 필수적인 것으로 알려져 있다. 본 연구에서는, 이러한 물질의 필요성이 신경의 배발생 중 insulin에서 Tf으로 바뀌어지며, 이 현상은 그 수용체 수준의 변화에 따라 나타나는 것임을 밝히었다. 또한, 신경아세포의 배양 시에도 세포 분화에 따라 insulin 수용체의 level은 급격하게 떨어지는 반면, Tf 수용체의 level은 증가하는 것으로 나타났다. 이러한 결과는, insulin과 Tf 수용체의 배발생-특이적 변화가 세포 배양 시에도 분화에 따라 재현됨을 의미한다. 세포 배양 시에 사용한 defined medium 자체 내에는 insulin과 Tf과 같은 물질이 존재하지 않기 때문에 insulin과 Tf은 신경 발생 조절의 신호 분자로 추측된다.