DEGENERATION OF LARGE MYOTUBES FOLLOWING REMOVAL OF TRANSFERRIN FROM CULTURE MEDIUM

EIJIRO OZAWA and YASUKO HAGIWARA

Division of Cell Biology, National Center for Nervous, Mental and Muscular Disorders, Kodaira, Tokyo 187, Japan

ABSTRACT

Chick myoblasts multiplied and formed large myotubes showing cross-striations and spontaneous twitching when incubated with a medium composed of Eagle's minimum essential medium, horse serum, and a small amount of chick transferrin (Tf). Large myotubes degenerated after Tf was removed from the medium. Fat droplets appeared in the cells within a few days and various changes took place. Most degenerated cells became detached from the culture dish within 1 or 2 weeks.

Myoblasts were relatively resistant to Tf deficiency. Large myotubes were formed again when the myoblasts were again incubated with Tf. These newly formed large myotubes quickly degenerated following the removal of Tf.

Chick myogenic cells do not grow in a medium composed of Eagle's minimum essential medium (MEM) and horse serum, but grow well when a small amount of chick embryo extract is added to the medium. Following this addition, myoblasts multiply and form large myotubes with cross-striations and spontaneous twitching (5, 13). Embryo extract can be replaced either by chick serum or by muscle trophic factor (MTF), which is a component of chick serum and embryo extract (5, 14). Very recently, MTF was identified in our laboratory as transferrin (Tf), an iron-binding protein in serum (8). We also found that Tf had class specificity in its muscle trophic effects (12), *i.e.*, chick and rat myogenic cells in primary culture do not grow in the absence of avian and mammalian Tfs, respectively, but do grow well in their presence. Therefore, horse Tf contained in the culture medium does not affect chick myogenic cell growth. Since Tf is also an indispensable component of serum-free culture medium for the culture of various kinds of mammalian cell lines (1, 2), the name 'muscle trophic factor' should be withdrawn and the substance should be referred to as transferrin, or Tf.

We have shown that Tf promotes myoblast

multiplication in proportion to its concentration, resulting in an increase in the number of nuclei contained in myogenic cells (14). We have found that large myotubes degenerated when cultured without chick Tf. Myoblasts and small slender myotubes, however, persisted for a fairly long period and grew into large myotubes following the readdition of chick Tf.

In this paper, we report on this process as observed under a light microscope. Parts of this work have been published in a preliminary report (10).

MATERIALS AND METHODS

Methods for chick Tf isolation, myoblast preparation from the 11th day chick embryo breast muscles, cell harvesting and creatine kinase (CK) assay have been described elsewhere (5, 11, 14).

The schedule of the experiments was as follows: 8×10^4 dissociated chick embryo breast muscle cells, purified by the differential adhesion technique, were inoculated onto 35 mm Falcon plastic Petri dishes precoated with gelatin. One hundred and four dishes were prepared. The cells were incubated with a medium composed of 85% MEM and 15% horse serum containing

Table 1 Concentration of Tf ($\mu\text{g/ml}$) in the Medium

	Days 4-8	Days 8-10	Days 10-15	Days 15-39
Group I	3	3	—	—
Group Ia	3	0	—	—
Group II	0	0	0	0
Group IIa	0	3	3	7.5
Group IIb	0	0	0	25

10 $\mu\text{g/ml}$ chick Tf; they were photographed and harvested for CK assay on selected days. (Each point shown in Fig. 1 denotes the mean of CK activity of 4 dishes.) On day 4, the dishes were divided into two groups (Table 1). The cells of group I and II were incubated further with and without 3 $\mu\text{g/ml}$ Tf, respectively. On day 8, group Ia was formed from group I by the removal of Tf and group IIa was formed from group II by the addition of 3 $\mu\text{g/ml}$ Tf. On day 15, group IIb was formed from group II by the addition of 25 $\mu\text{g/ml}$ Tf. On the same day, the Tf concentration in group IIa was raised to 7.5 $\mu\text{g/ml}$. The medium of all the groups was changed every 3 or 4 days.

RESULTS

On day 4, CK activity in the myogenic cells was detectable (Fig. 1). This activity increased in group I until day 8, and then decreased slightly, whereas the activity in group Ia decreased substantially with time. The CK activity in group II increased slightly from day 4 to day 6, but then decreased to an almost undetectable level by day 15. This temporal increase in CK activity was

sometimes but not always observed. The difference in CK activity between groups I and II was not significant on day 5, but was on day 6, with Student's *t*-test at the 5% level. In group IIa, the activity increased substantially after the readdition of Tf following a certain lag time. The difference between group II and IIa was significant on day 15. The activity in group IIa further increased to reach a plateau level on day 19. In group IIb, the activity did not seem to have increased for more than a week, as deduced from microscopical observation of the cells, but had reached a fairly high level by day 31, and became even higher on day 39. CK activity in group II remained undetectable during these periods.

Large myotubes had formed by day 4 (Fig. 2A). The myotubes in group I continued to grow, and some of them showed cross-striations and spontaneous twitching by day 6, when the cells were confluent in the dish. The cross-striations became more remarkable with time (Fig. 2B). The cells did not change much in appearance from day 8 to 10, although a small number of myotubes were detached.

The large myotubes in group Ia were detached (Fig. 3B) or degenerated on day 9 and thereafter. The detachment of the myotubes corresponded with a considerable decrease in CK activity (Fig. 1).

Myoblast fusion in group II seemed to have progressed by day 6, as judged from successive observations (Fig. 4, A and B). The large myotubes looked flat and contained numerous tiny spots and many small opaque areas, whose diameters were approximately 1 and 10 μm , respec-

Fig. 1 Time course of CK activity accumulated in myogenic cells on the dishes. For further details, see Materials and Methods and Table 1.

Fig. 2 Myogenic cells in group I. A: incubated for 4 days; B: 8 days. Successive observations of the same field. $\times 150$

Fig. 3 Myogenic cells in group Ia. A: incubated for 8 days; B: 10 days. Tf was removed on day 8. Note that large myotubes have disappeared by day 10. Successive observations of the same field. $\times 150$

Fig. 4 Myogenic cells in group II. A: incubated for 4 days when Tf was removed; B: 6 days; C: 10 days; D: 14 days. Opaque areas are shown by arrows in B and C. Successive observations of the same field. $\times 150$

Fig. 5 Fat degeneration appearing in the cells in group II. A: cells in group I; B: cells in group II incubated for 7 days. Cells were fixed with 2.5% glutaraldehyde buffered with 100 mM sodium cacodylate and stained by Oil Red O. Cells in group II were heavily stained, but only a few stained granules were scattered in the cells in group I. $\times 375$

Fig. 6 Other types of myotube degeneration in group II. A: spheroid formation. Cells were incubated for 7 days. $\times 220$. B: short thorny projections and twisting of the cell body. Cells were incubated for 15 days. $\times 110$. Tf was removed on day 4 in all cases.

tively. The tiny spots were fat droplets, since they were stained by Oil Red O (Fig. 5). These cells became thinner, and the opaque areas grew or joined to form a demarcated area where the cell body looked extremely thin. One of the myotubes shown in Fig. 4C was disrupted and half of it was lost by day 10. Most large myotubes on the dishes disappeared by day 14, leaving some mononucleated cells and small slender myotubes intact (Fig. 4D), but these also finally disappeared.

Other kinds of degenerative changes were as follows. Numerous vacuoles appeared in the myotubes and these often joined and broke the myotubes into pieces. Tiny spheroids appeared on the cell surface (Fig. 6A). They were outside the cell body, since these spheroids and the cell body were not in focus simultaneously. Their size usually ranged from 5 to 40 μm in diameter. The myotubes became thin and sometimes collapsed after the spheroids were formed. Sometimes these spheroids left the

cell body and floated in the medium; granular structures were often seen in the floating spheroids. The nuclei in the cells with spheroids shown in Fig. 6A looked condensed, and such nuclei were often found in cells with advanced degenerative changes. The myotube edges were sometimes very ragged with short thorny projections. These usually appeared at the site where the cell was atrophic, making it appear twisted (Fig. 6B).

Group IIa was formed from group II by the addition of Tf to the medium on day 8 (Fig. 7A). Most degenerated cells collapsed and became detached soon after the addition (Fig. 7B). The myoblasts and small slender myotubes, however, remained relatively normal in appearance (Fig. 7C). On day 10, there were numerous large and small slender myotubes and numerous undetermined cells, presumably myoblasts and fibroblasts (Fig. 7D). Some of the large myotubes grew larger and showed cross-striations and spontaneous twitching.

Fig. 7 Myogenic cells in group IIa. A: incubated for 8 days when Tf was readded to the medium; B: 9 days; C: 11 days; D: 19 days. Successive observations of the same field. Note that degenerated cells disappeared after the readdition of Tf. $\times 150$

In group II, there were a small number of myotubes on day 15. Many of them were very thin and wide, and their intracellular structures were scarcely recognizable except for randomly scattered nuclei. Myoblasts and small slender myotubes were also seen with or without slight deformities. These cells were finally destroyed and became detached from the dish when they were incubated without Tf.

Some other changes were observed in group IIb. Many long arms protruded from flat myotubes (Fig. 8, A and B), which were not observed before the readdition of Tf. Myoblasts attached to flat cells in other cases (Fig. 8C); presumably, some of these flat cells would be taken into newly formed large myotubes.

The myoblasts formed myotubes after a time-lag of several days following addition of Tf (Fig. 9, A and B) and then grew rapidly (Fig. 9C). These newly formed large myotubes degenerated quickly when Tf was again removed from the medium. Some cells degenerated even in the presence of Tf, while other large myotubes were formed at the same time in the same dish. The most common type of degeneration was flattening. These flat cells contained large vacuoles and then were destroyed.

DISCUSSION

CK activity derives primarily from myotubes (6, 17, 18), and thus such activity can be interpreted as expressing the quantity of myotubes present in a culture dish (11). Fig. 1 shows that CK activity is increased and decreased in the presence and absence of chick Tf in the medium, respectively, and this can thus be interpreted as an increase and a decrease in the quantity of myotubes.

Removal of Tf from the medium resulted in severe morphological changes and the final detachment of most large myotubes. The first change was fat degeneration (Fig. 5), which lasted for a long period. Decrease in thickness of the cell body was also an early and conspicuous change. A tentative classification of subsequent changes was made. These changes took place either simultaneously or sequentially in a dish or even in a single myotube.

Type 1, general atrophy There were two kinds of changes: decrease in width and thickness of parts or all the cell body (Fig. 6B), and extreme flattening of the cell body (Fig. 8, A and B).

Type 2, vacuolation

Type 3, myotube detachment (Fig. 3B) The

Fig. 8 Various types of reaction following the readition of Tf in group IIB. Tf was added to the medium on day 15. A: flat myotubes with numerous nuclei and long projections on day 18. B: flat myotubes with a few nuclei and long projections on day 28. C: myoblasts attached to a myotube on day 21. $\times 150$

detaching cells usually suffered from severe fat degeneration, and either deformation by spheroid formation or no deformation occurred.

Type 4, spheroid formation (Fig. 6A) The spheroid may have been cytoplasm leaking from the cell body, since the cells became thin and collapsed, and the spheroids sometimes contained granular structures. Two types of similar changes have been described in other kinds of cells. One is called 'zeiotic blebs', in which a relatively rapid projection of the cytoplasm occurs (4, 15). The other involves blisters consisting of the cell membrane and a clear fluid

Fig. 9 Myogenic cells of group IIB. A: myoblasts showed slight deformities by day 9, when the cells had been incubated without Tf for 5 days. Tf was readded to the medium on day 15. B: small myotubes reformed by day 21. C: the myotubes grew by day 24. Successive observations of the same field. $\times 150$

(15); these sometimes leave the cell body and float into the medium. The present spheroids seem to be blisters, judging from the light microscopic photographs cited in the paper and the fact that they float into the medium. But the spheroids might be zeiotic blebs, since they seem to contain cytoplasm. Further study is necessary for a final conclusion.

Type 5, projection formation (Fig. 6B)

In contrast to large myotubes, myoblasts are relatively resistant to deficiency of Tf and fuse easily upon its removal. This may be explained by the line of reasoning described by Yaffe, *i.e.*,

that reduction of embryo extract in culture medium promotes myogenic cell fusion (19). Myoblasts and small slender myotubes form large myotubes after the readdition of Tf. These newly formed myotubes are formed from cells that are resistant to Tf deficiency but are susceptible to degeneration due to the Tf deficiency. Fibroblasts are fairly resistant to Tf deficiency, but finally disappear.

One might argue that the pathologic changes described here may also be found in culture of myogenic cells made under the conditions widely used in many laboratories. This is true, but such changes, if any, can be found far less frequently in the usual cultures than in the present case, in which almost all the large myotubes degenerated.

It has been assumed that the role of Tf in myoblast multiplication is to give iron to the cells (6). In the present study, chick myotubes were shown to require chick Tf in order not to degenerate. Horse Tf contained in the culture medium was not effective. This can be understood when the class specificity of Tf is taken into consideration (12). Ferric ion at concentrations about 200 times higher than the concentration of iron bound to chick Tf was effective in growing chick myogenic cells (6). We also found that ferric ion at the same concentrations as described above was effective in keeping large myotubes intact (Ozawa *et al.* unpublished data). Therefore, it is possible that the degenerative process described in this paper is due to deficiency of iron in myotubes.

Florini and Roberts (3) have reported that Yaffe's L₆ cells (16), a rat myogenic cell line, and rat myogenic cells in primary culture grew in a serum-free medium containing a high concentration of fetuin (about 1 mg/ml). They have further showed that Tf has no effect on the myogenic cells when added to the medium, and insisted that Tf was not necessary for myogenic cell growth. The present report as well as our previous ones (8, 12, 13, 14), showed that homo-class Tf was necessary for chick or rat myogenic cell growth. The discrepancy can be explained if we adopt Barnes and Sato's opinion (1) on this problem that an impurity in the fetuin preparation acts in a way similar to Tf. Indeed, one of the commercially available fetuins (Deutsche type, GIBCO Laboratories) showed a large number of bands on SDS-gel electrophoresis which included a band very similar to Tf (unpublished data by I. Ii).

We wish to express our cordial thanks to Professor S. Ebashi for his valuable advice and discussions. Our thanks are also due to Professor A. Asoda for his encouragement. This work was partially supported by grants from the National Center for Nervous Mental and Muscular Disorders (NCNMMD) of the Ministry of Health and Welfare, the Ministry of Education, Science and Culture, Japan, and the Okinaka Memorial Institute for Medical Research.

Received for publication 19 October 1981; and in revised form 26 December 1981

REFERENCES

1. BARNES D. and SATO G. (1980) Serum-free cell culture: a unifying approach. *Cell* **22**, 649–655
2. BARNES D. and SATO G. (1980) Methods for growth of cultured cells in serum-free medium. *Analyt. Biochem.* **102**, 255–270
3. FLORINI J. R. and ROBERTS S. B. (1980) A serum-free medium for the growth of muscle cells in culture. *In Vitro* **15**, 983–992
4. GODMAN G. C., MIRANDA A. F., DEITCH A. D. and TANENBAUM S. W. (1975) Action of cytocharasin D on cells of established lines. III. Zeiosis and movement at the cell surface. *J. Cell Biol.* **64**, 644–667
5. HAGIWARA Y., KIMURA I. and OZAWA E. (1981) Chick embryo extract, muscle trophic factor and chick and horse sera as environments for chick myogenic cell growth. *Develop. Growth Differ.* **23**, 249–254
6. HASEGAWA T., SAITO K., KIMURA I. and OZAWA E. (1981) Fe³⁺ promotes *in vitro* growth of myoblasts and other cells from chick embryos. *Proc. Japan Acad.* **57**, Ser. B, 206–210
7. Ii I., KIMURA I., HASEGAWA T. and OZAWA E. (1981) Transferrin is an essential component of chick embryo extract for avian myogenic cell growth *in vitro*. *ibid* **57**, Ser. B, 211–216
8. KIMURA I., HASEGAWA T., MIURA T. and OZAWA E. (1981) Muscle trophic factor is identical to transferrin. *ibid* **57**, Ser. B, 200–205
9. OHTSUKI I. and OZAWA E. (1977) Difference in saponin sensitivity between myotubes and mononucleated cells from chick breast muscle. *Cell Struct. Func.* **2**, 367–370
10. OZAWA E. (1977) Trophic effects on chick muscle cells of a factor promoting chick myoblast multiplication. *Proc. Japan Acad.* **53**, Ser. B, 130–132
11. OZAWA E. (1978) Differences in sensitivity to Ca ion lack between myoblasts and large myotubes from chicken breast muscle. *Develop. Growth Differ.* **20**, 179–189
12. OZAWA E. and HAGIWARA Y. (1981) Avian and mammalian transferrins are required for chick and rat myogenic cell growth *in vitro* respectively. *Proc. Japan Acad.* **57**, Ser. B, 406–409
13. OZAWA E. and KOHAMA K. (1973) Partial purification of a factor promoting chicken myoblast

- multiplication *in vitro*. *Proc. Japan Acad.* **49**, 852-856
14. OZAWA E. and KOHAMA K. (1978) Muscle trophic factor: I. Assay of a muscle trophic factor by measurement of muscle cell nuclei. *Muscle and Nerve* **1**, 230-235
 15. PRICE Z. H. (1967) The micromorphology of zeiotic blebs in cultured human epithelial (HEp) cells. *Exp. Cell Res.* **48**, 82-92
 16. RICHER C. and YAFFE D. (1970) The *in vitro* cultivation and differentiation capacities of myogenic cell lines. *Develop. Biol.* **23**, 1-22
 17. SHAINBERG A., YAGIL G. and YAFFE D. (1970) Control of myogenesis *in vitro* by Ca^{2+} concentration in nutritional medium. *Exp. Cell Res.* **58**, 163-167
 18. SHAINBERG A., YAGIL G. and YAFFE D. (1971) Alterations of enzymatic activities during muscle differentiation *in vitro*. *Develop. Biol.* **25**, 1-29
 19. YAFFE D. (1971) Developmental changes preceding cell fusion during muscle differentiation *in vitro*. *Exp. Cell Res.* **66**, 33-48