Basis for the Assay of Myogenic Cell Growth In Vitro
Using Creatine Kinase Activity as an Index, with Special
Reference to Measurement of Power Ratio of
Transferrins in Growth Promotion

Yasuko HAGIWARA, Tadashi SHIMO-OKA*, Kazuo OKAMURA**
and Eijiro OZAWA
Division of Cell Biology, National Institute of Neuroscience,
National Center of Neurology and Psychiatry, Kodaira, Tokyo 187, Japan

Present address:
* Tissue Culture Laboratory, Iwaki Glass Company,
Funabashi, Chiba 273, Japan
** Seikagaku Kogyo Company, Higashi-yamato,
Tokyo 189, Japan

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Abstract—In order to establish an assay for the in vitro growth of myogenic cells, we
investigated whether creatine kinase (CK) activity can be used as an index for the
estimation of growth. The content of CK activity in a primary myogenic cell culture,
almost all of which originated from myotubes, was shown to be linearly proportional
to the content of proteins in the myotubes and increased concomitantly with myotube
growth. CK activity can be easily and accurately assayed and used for routine
assay of the cell growth. As an example of the application of CK activity for the
bioassay of growth promoting substances, the relative potency (Pr) of turkey
transferrin (Tf) to chick Tf in the promotion of chick myogenic cells was examined
with a parallel line assay. The calculation limited that Pr = 0.363, ranging from
0.356 to 0.370, at 95% safety. The results demonstrated the usefulness of CK
activity for estimating cell growth, as well as the value of a statistical method for
the assay of a growth factor in vitro.

To facilitate the study of effects of growth factors, it is a prerequisite to establish a simple
and reliable bioassay method that can be performed in vitro. For this purpose, an accurate
estimation of the “amount” of cells present on a culture dish is most important, since
growth rate is the increase in the “amount” in a given period. Indices of cell proliferation
such as cell number, DNA content, thymidine incorporation into DNA and so forth are
widely used as parameters, since the growth of cultured cells is usually defined as the in-
crease in cell number in a certain period. In the case of myogenic cells in culture, especial-
ly in primary cell culture, however, the situation is different. Myoblasts proliferate, then
fuse to form myotubes which lose the ability
to proliferate (1). The number of cells de-
creases on fusion, although the amount of
DNA does not change. The multinuclear cells
continue to enlarge their cell bodies by ac-
cumulating various kinds of proteins, es-
specially muscle specific proteins such as
creatine kinase (CK) (2, 3). Enlargement of
cells is also considered to be growth. There-
fore, it is reasonable to use the amount of
protein accumulated in myotubes as a pa-
rameter of growth.

CK has long been known to be contained
fairly specifically in muscle cells. Reporter et
al. (2) showed that muscle cells accumulate
the enzyme as embryos grow in vivo. Shainberg et al. (4) and Turner et al. (5)
showed that myogenic cells accumulate the enzyme rapidly as soon as cells fuse in vitro.
On these bases, CK activity has been used as
an index to estimate myogenic cell growth
(6); Why it can be used as a quantitative
index, however, has not been clear.
In this paper, we show evidence that CK activity represents the amount of protein contained in myotubes, and thus the "amount" of myotubes. As an example of its application, the power ratio of turkey transferrin (Tf) to chick Tf in promotion of myogenic cell growth was estimated by parallel line assay using CK activity as a growth index, and it was compared with the value derived from intuitive observation of dose-response curves. Parallel line assay has long been used to estimate the relative potency (Pr) of a test preparation to the potency of a standard preparation.

Materials and Methods

Cell culture: Myoblasts were prepared from the breast muscle of 11 day-old chick embryos as described previously (7). A specified number of myoblasts was inoculated to a gelatin-coated plastic culture dish (Ø: 35 mm) with 2.5 ml of basal culture medium (BCM) composed of 85% Eagle's MEM (Nissui, Tokyo) and 15% horse serum (Gibco, Grand Island) containing a specified amount of chick or turkey Tf, and incubated in a water-saturated mixture of 95% air and 5% CO₂. In BCM, chick cells do not grow if avian Tf is not added (8). Cells were harvested in the following manners. In the comparison of protein concentration with CK activity, cells on the dish were washed twice with 0.9% NaCl solution containing 1 mM CaCl₂ and then frozen with 1.2 ml H₂O at -80°C for 1 hr. After thawing, the cell suspension was collected and the cell debris spun down. From the supernatant solution, an aliquot (20 µl) was removed for the CK activity assay. Then, NaOH was added to the original solution to a final concentration of 0.1 N in order to resolve the precipitate for protein measurement. In other cases, cells were washed and lysed with 1% Triton X-100. After the cell debris was removed by centrifugation, CK activity of the supernatant solution was assayed. The activity showed little dependence on the solution or the cell collection method used.

Sample preparation and assay: Chick and turkey Tf were prepared from egg white and serum, respectively, as described previously (9).

CK activity was assayed with a MARK II autoanalyzer (LKB, Bromma) using a CK-NAC kit (Boehringer, Mannheim). Protein was measured by the method of Lowry et al. (10).

Statistical calculations: All the calculations for parallel line assay were performed after Finney (11).

Results

Chick myotubes were cultured with various concentrations of chick Tf, which were practically free from fibroblasts by treatment with Ara C (final concentration: 5 µM) on day 4 after inoculation. They were harvested on day 5 to assay the amount of protein and CK activity. A linear correlation was found between these two parameters, although the line did not exactly pass through the original point (Fig. 1). The correlation coefficient ranged from 0.99 to 0.96 in repeated experiments.

Fibroblasts were collected from primary myogenic cells passed twice after myotube
formation. They were cultured for 3 days to make them almost confluent. CK activity content was 0.12U, for example, even though a small number of tiny myotubes were found in the culture. This value was far less than the activity contained in the myotubes (several units per dish, see Fig. 2). Therefore, CK activity derived from fibroblasts concomitantly present in myogenic cell culture is assumed to be very low and can be neglected.

The time courses of morphological changes in myogenic cells and CK activity accumulation were compared (Fig. 2). In the presence of chick Tf (15 μg/ml), myotubes grew with the lapse of time, and CK activity was accumulated concomitantly. At the same time, morphological growth of myotubes was observed. In the absence of chick Tf, neither myotube growth nor CK accumulation took place (9). An intuitive parallelism between CK accumulation and morphological growth was always observed.

Myogenic cells were inoculated to 192 dishes and cultured under the same conditions. After 5 days, cells were harvested and CK activities were assayed. A histogram of these activities is shown in Fig. 3. The cumulative distribution of these data was plotted on probability paper (Fig. 4). Because of its linearity, the population was estimated to have

![Fig. 2. Comparison of CK activity accumulation (A) and morphological changes (B).](image-url)
a normal distribution, and the data derived from these methods are amenable to analysis of variance. The mean and standard deviation (SD) were 1.564 and 0.1057 U/dish, respectively. Their coefficient of variance (CV=SD/mean) was 6.8%.

Myogenic cells were cultured in BCM containing various concentrations of chick or turkey Tf for 4 days. CK activities were assayed and plotted against the Tf concentrations (Fig. 5). Each curve was typically sigmoidal. The concentrations of chick and turkey Tf required to give half maximum (ED50) were 3 and 10 µg/ml, respectively.

Out of the data shown in Fig. 5, three points each from the chick Tf (1.5, 3, 6 µg/ml) and turkey Tf (3, 6, 12 µg/ml) curves were selected for parallel line assay. The CK activities of the corresponding Tf concentrations are shown in Table 1. On the basis of these parameters, tables of analysis of variance were made according to Finney (ref. 11: see Table 5.2.1, 5.2.2 and 5.2.3 of the book) (Tables 2 and 3). For the strict application of the assay method, the mean square of the regression should be greater and those of parallelism, quadratic and differences of quadratic should be smaller than the mean square of the error (11). In the present data, the mean square of the regression was much larger and the mean square of quadratic was smaller than that of the error. As for parallelism, $F_{0.25} = 1.41 < F = 2.60 < 3.01 = F_{0.01}$ (11). Therefore, it was roughly assumed that both the lines are linear and parallel. On that as-
Assay of Myogenic Cell Growth

Table 1. Response metarneters in an assay of transferrin

| Dose   | Standard preparation | Chick Tf (μg/ml) | Test preparation | Turkey Tf (μg/ml) |
|--------|----------------------|-----------------|------------------|------------------|
| 1.5    | 1.360                | 2.374           | 3.303            | 803              |
| 3      | 346                  | 605             | 3                 | 200              |
| 6      | 335                  | 592             | 3                 | 205              |
| 12     | 337                  | 557             | 3                 | 196              |
|        | 342                  | 620             | 3                 | 202              |

Table 2. Analysis of variance

| Nature of variation   | d.f.* | Sum of squares | Mean square |
|-----------------------|-------|----------------|-------------|
| Preparations          | 1     | 87,967         | 87,967      |
| Regression            | 1     | 1,100,926      | 1,100,926   |
| Parallelism           | 1     | 6,045          | 6,045       |
| Quadratic             | 1     | 2,228          | 2,228       |
| Difference of quadratics | 1   | 5,146          | 5,146       |
| Between doses         | 5     | 1,202,348      | 240,469     |
| Error                 | 18    | 41,765         | 2,320       |
| Total                 | 23    | 1,244,113      |             |

*df*: degree of freedom.

Table 3. Coefficients of orthogonal constants for the (3,3) design

| Dose   | S1   | S2   | S3   | T1   | T2   | T3   | Divisor | Sum  |
|--------|------|------|------|------|------|------|---------|------|
| Response Total | 1,360| 2,374| 3,303| 803  | 1,724| 3,057| 24      | 12,621|
| Lp     | -1   | -1   | -1   | 1    | 1    | 1    | 24      | -1,453|
| L1     | -1   | 0    | 1    | -1   | 0    | 1    | 16      | 4,197 |
| L1*    | 1    | 0    | -1   | -1   | 0    | 1    | 16      | -311  |
| L2     | 1    | -2   | 1    | 1    | -2   | 1    | 32      | -327  |
| L2*    | -1   | 2    | -1   | 1    | -2   | 1    | 32      | 497   |

Discussion

Myogenic cells grow by accumulating proteins, and protein must be the actual index for muscle cell growth. Using the index for routine work, protein determination is rather troublesome. One of the reasons for this is that protein is also derived from fibroblasts which inevitably contaminate the primary myogenic cell culture. CK activity which is fairly specifically present in muscle cells was examined to determine whether it can be used in place of protein amount to express growth. In the

assumption, calculation of the Pr of turkey Tf to the potency of chick Tf was performed according to Finney (11). Means of the standard and test samples were \( Y_s = 586.42 \) and \( Y_r = 465.33 \), respectively, and the regression coefficient was \( b = 262.31 \). Therefore, the estimated log potency \( M = \frac{(Y_s - Y_r)}{b} = -0.4616 \). Since experiment was designed so that the concentrations of Tf's in the standard and test solutions increased by a factor of 2, \( M \) was multiplied by \( \log_{10} 2 \) to convert \( M \) into logarithms to base 10. Thus, Pr of turkey Tf = 3/6 antilog 1.8611 = 0.363. The confidence limits at 95% safety due to Fieller's equation (explained in 11) ranged from 0.356 to 0.370.
In the present study, CK activity was linearly parallel to protein amount in a primary myogenic cell culture devoid of fibroblasts. Therefore, CK activity can be used as an index for myogenic cell growth.

The enzyme assay of CK activity can be performed easily and accurately. CV of CK activity of cultured muscle is quite low, and this method can be applied for the quantitative study of myogenic cell growth. The data thus obtained was amenable to analyses by usual statistical methods.

Dose-response curves of chick and turkey Tf's are shown in Fig. 5. When the potency of chick Tf to promote cell growth is put one unit, the potency of turkey Tf seems to be about 3.3 by intuitive observation. When the data in Table 1 derived from Fig. 5 were analyzed by (3.3) design parallel line assay methods (11), Pr was 0.363, suggesting that the intuitive observation gave a value close to the calculated value.

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