Erythrocyte-mediated microinjection, a technique to study protein degradation in muscle cells

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The technique of erythrocyte-mediated microinjection has been successfully adapted for use with cultured muscle cells. Erythrocytes were fused with primary chick myotube cultures with poly(ethylene glycol), and fluorescent antibodies to haemoglobin demonstrated that this protein was injected into the sarcoplasm of myotubes. The microinjection treatment did not significantly alter protein metabolism in the muscle cells as monitored by rates of synthesis and degradation of muscle proteins. ^{125}I -labelled ribonuclease A and bovine serum albumin were degraded with the expected exponential decay kinetics after microinjection into muscle cells, and the half-life of ribonuclease A (40 h) was approximately twice that of bovine serum albumin (17 h). The degradation of ribonuclease A in the muscle cells was enhanced 1.6-fold in the absence of horse serum and chick-embryo extract, whereas the degradation of bovine serum albumin was not altered during deprivation. These results are characteristic of the breakdown of microinjected ribonuclease A and bovine serum albumin in other cell types. Therefore, our experiments indicate the erythrocyte-mediated microinjection is a valid technique to study protein degradation in primary chick muscle cultures.

Cellular proteins are continuously degraded to their constituent amino acids and replaced by new synthesis (Goldberg & Dice, 1974; Goldberg & St. John, 1976). The mechanisms and regulation of intracellular proteolysis are not completely understood, despite intensive study for the past 15 years (Amenta & Brocher, 1981; Herskho & Ciechanover, 1982; Mortimore, 1982). Several groups have applied the technique of erythrocyte-mediated microinjection to study protein degradation. Microinjection does not alter protein metabolism in recipient fibroblasts (Hendil, 1980; Neff *et al.*, 1981) and has been used to introduce proteins such as haemoglobin, bovine serum albumin, antibodies, non-histone chromosomal proteins, ubiquitin and several enzymes into the cytoplasm of cultured cells (Kulka & Loyter, 1979; Zavortink *et al.*, 1979; Hendil, 1980; Neff *et al.*, 1981). Results from such

microinjection studies have helped to clarify the alterations in proteolysis associated with cellular senescence (Dice, 1982) and to assess the relative contributions of lysosomal and non-lysosomal pathways in the catabolism of intracellular proteins (Bigelow *et al.*, 1981; Chin *et al.*, 1982). Furthermore, microinjection has permitted detailed analyses of the influence of protein structure on intracellular half-lives (Backer *et al.*, 1983).

Erythrocyte-mediated microinjection has been successfully applied to several cell types in culture (Schlegel & Rechsteiner, 1978; Kulka & Loyter, 1979), but has not previously been reported for muscle cells. Elucidation of the mechanisms and regulation of protein catabolism in these cells is important physiologically, since skeletal muscle accounts for over 50% of the total body protein and serves as a supply of amino acids during nutritional deprivation (Goldberg & Dice, 1974; Felig, 1975; Ruderman, 1975). We report here that primary cultures of chick skeletal muscles can be microinjected with specific proteins and the erythrocyte-mediated microinjection is a valid approach for the study of intracellular protein degradation in these cells.

Abbreviations used: CEE, chick-embryo extract; RNAase A, bovine pancreatic ribonuclease A; PEG, poly(ethylene glycol); HBSS, Hanks (1948) balanced salt solution; MEM, minimal essential medium; phosphate buffered saline, 72 mM- Na_2HPO_4 /28 mM- NaH_2PO_4 /26 mM- NaCl , pH 7.2; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

Methods and materials

Tissue culture

Cells were isolated from 11- or 12-day-embryonic-chicken breast muscle (SPAFAS, Norwich, CT, U.S.A.) by mechanical dissociation as described previously (McElligott *et al.*, 1981). Cultures were plated at a density of $(1.5-3) \times 10^6$ cells/60mm-diameter dish. Culture plates were collagen-coated with Vitrogen 100 (Flow Laboratories, McLean, VA, U.S.A.). Cells were grown in medium containing 87% minimal essential media (MEM), 2% chick-embryo extract (CEE), 10% heat-inactivated horse serum and 1% penicillin/streptomycin (all from Grand Island Biological Co., Grand Island, NY, U.S.A.) in a humidified atmosphere of CO₂/air (1:9) at 37°C. Cytosine arabinoside (10 µM, final concn.) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to cultures for 24–48 h after myoblasts had fused (day 2 or 3 after plating). Cultures were used for microinjection experiments between the fourth and eighth day in culture.

Radioiodination of proteins

Bovine serum albumin (fraction V) (Miles Laboratories, Elkhart, IN, U.S.A.) and RNAase A type XII-B (Sigma Chemical Co., St. Louis, MO, U.S.A.) were iodinated by using lactoperoxidase and glucose oxidase as described previously (Neff *et al.*, 1981). By using this procedure, we routinely observe iodine/protein molar ratios of 0.5.

Radioactivity (c.p.m.) in monolayer at end of experiment + total acid-soluble radioactivity (c.p.m.) released into medium = Total radioactivity (c.p.m.) microinjected into muscle cells (1)

[Total radioactivity (c.p.m.) microinjected – acid-soluble radioactivity (c.p.m.) in medium/total radioactivity (c.p.m.) microinjected]_t × 100 = Percentage of radioactivity remaining in cells at time *t*

Loading and fusion

Ghosts were prepared and loaded with radioactive proteins as described previously (Neff *et al.*, 1981). Koch–Light PEG 1000 (Research Products International Corp., Elk Grove Village, IL, U.S.A.) was autoclaved and diluted to a final concentration of 40% with MEM (PEG solution). We have observed that each new lot of PEG must be tested to determine the optimal concentration of PEG required for fusion. With this lot of PEG, 40% (w/v) PEG gave maximal fusion without deleterious effects on the myotubes (see Figs. 2 and 3 below). To achieve fusion, the muscle cultures were first washed with 5 ml of HBSS (Grand Island Biological Co., Grand Island, NY, U.S.A.). Then $(3-6) \times 10^8$ loaded ghosts suspended in 0.05 ml of HBSS were added to each plate, and the plate was tilted to distribute the ghosts evenly over the surface. The PEG solution

(0.35 ml) was pipetted on to the plate in small droplets over the surface, and the plate was tilted to spread the PEG solution. After 1 min, 4.5 ml of MEM were added to each plate with minimal disturbance of the PEG layer. The cells were immediately washed with 15 ml of HBSS containing 5% (v/v) foetal-calf serum to remove non-fused ghosts. The washing was most efficient if plates were held vertically and the wash medium ejected from the pipette in sweeping motions from top to bottom of plate. The wash medium and non-fused ghosts were removed from the bottom of the plate by continuous suction. The cultures were then incubated for 4 h in MEM containing serum and CEE before beginning degradation measurements. The medium was removed and replaced with either MEM containing CEE and horse serum or MEM alone and a portion of medium was sampled to determine background radioactivity at zero time.

Measurements of degradation of microinjected proteins

The degradation of microinjected proteins was determined by measuring the acid-soluble radioactivity released into the medium as described previously (Neff *et al.*, 1981). Portions (0.5 ml) were taken from culture plates that originally contained 6 or 8 ml of medium. After correcting for volume changes due to successive sampling, the following calculations allowed construction of degradation curves:

Rates of cellular protein synthesis and degradation

Protein synthesis and degradation were measured in two groups of cell cultures. One group was microinjected with unlabelled bovine serum albumin and the control group was subjected to media changes without microinjection. Protein synthesis was measured at various times after microinjection by labelling cells in MEM supplemented with 10% (v/v) horse serum, 2% CEE, 2 mM-unlabelled tyrosine and 5 µCi/ml of [³H]tyrosine (30–50 Ci/mmol; New England Nuclear Corp., Boston, MA, U.S.A.). After 1 h, cells were washed with HBSS containing 2 mM-unlabelled tyrosine and collected in 10% (w/v) trichloroacetic acid. The acid-precipitable pellet was dissolved in 0.1 M-NaOH/0.1% sodium deoxycholate and counted for radioactivity in Solusciint A fluor (National Diagnostics, Somerville, NJ, U.S.A.) with a Packard model 3255

liquid-scintillation counter. Quenching was monitored with an external standard. The protein content was determined by using the procedure of Lowry *et al.* (1951).

To determine the effect of microinjection on the degradation of endogenous muscle proteins, cellular proteins were labelled for 2 days in MEM supplemented with serum and CEE and 0.5 μ Ci of [14 C]tyrosine (>450 mCi/mol; New England Nuclear)/ml. This medium was removed and replaced with medium containing 6 μ Ci of [3 H]tyrosine/ml for 2 h to label short-lived proteins. The labelling medium was then removed and the cells were microinjected with unlabelled RNAase A. The cell cultures were incubated in media containing 2 mM non-radioactive tyrosine and samples of the medium were taken at various times. The trichloroacetic acid-soluble radioactivity was monitored as a measure of degradation of short-lived (3 H-labelled) and long-lived (14 C-labelled) proteins. Degradation curves were constructed as described above for catabolism of microinjected proteins.

Fluorescent localization of microinjected haemoglobin

To localize haemoglobin that had been microinjected into myotubes, a fluorescent antibody to human haemoglobin was used (fluorescein-conjugated IgG fraction of rabbit anti-(human haemoglobin) (Cappel Laboratories, Cochran, PA, U.S.A.). Erythrocytes were rinsed three times in HBSS, diluted to a 30% solution and used for microinjection. Within 5 min after fusion, the myotubes were rinsed with phosphate-buffered saline at 37°C and fixed in 3% formaldehyde in phosphate-buffered saline. After 30 min at room temperature the cells were rinsed with phosphate-buffered saline and incubated for 10 min in phosphate-buffered saline containing 0.1 M-glycine. Cultures were again rinsed with phosphate-buffered saline and incubated in phosphate-buffered saline

containing 0.1% Triton X-100 for 2 min. After a further wash with phosphate-buffered saline, a 1:100 dilution of antibody was added to cells at 37°C for 45 min. Cells were rinsed three times with phosphate-buffered saline and photographed with a Zeiss photomicroscope III equipped with epifluorescence and phase-contrast optics. The objective was a 40 $\times$ water-immersion lens (numerical aperture 0.75).

Results and discussion

Our results indicate that erythrocyte-mediated microinjection may be used to study the degradation of specific proteins in muscle cultures. The technique is relatively rapid and allows the introduction of sufficient labelled protein into the myotube cultures to accurately measure protein degradative rates.

Preliminary studies

Our previous studies (Neff *et al.*, 1981) showed that: (1) RNAase A can be iodinated with up to three iodine atoms/enzyme molecule without effect on enzymic activity or susceptibility to proteolytic attack *in vitro*. Furthermore, 125 I-labelled RNAase A and 3 H-labelled RNAase A (labelled by reductive methylation) have identical half-lives after microinjection into fibroblasts (Backer *et al.*, 1983), suggesting that neither labelling procedure seriously disrupts RNAase A structure. In contrast, a single iodine atom added to bovine serum albumin increases its susceptibility to proteolytic attack *in vitro* by 3-fold, which indicates that bovine serum albumin is structurally altered by iodination. (2) Neither RNAase A nor bovine serum albumin is degraded within erythrocyte ghosts on the basis of production of acid-soluble radioactivity or altered electrophoretic mobility in SDS/polyacrylamide gels. (3) RNAase A and bovine serum albumin appear to be loaded into the interior of erythrocyte ghosts without significant binding to the erythro-

Table 1. *Amount of haemoglobin, RNAase A and bovine serum albumin microinjected into chick myotubes*

The following information was used to calculate the results given below. Normal human erythrocytes contain 29 pg of haemoglobin tetramer per cell and the haemoglobin tetramer has M_r 64000. We assumed that each myotube was microinjected with a single erythrocyte, since 50–70% of the myotubes were not detectably injected at all (Fig. 1). The concentration of RNAase A and bovine serum albumin loaded into ghosts was computed from the specific radioactivity of the labelled proteins (approx. 10^6 c.p.m./ μ g in each case) and the amount of radioactivity loaded into the ghosts (15000 c.p.m./ 10^6 ghosts for the RNAase A and 7000 c.p.m./ 10^6 ghosts for bovine serum albumin). The amount of protein per myotube was calculated by assuming an initial plating density of 2.25×10^6 cells/plate, a doubling of the myoblasts before fusion, and fusion of 20 myoblasts to form a myotube. This would give 2.25×10^5 myotubes per plate, and the measured amount of protein per plate is about 1 mg. Therefore each myotube contains 4.4 ng of protein.

Protein	Amount per erythrocyte	Ghosts fused per myotube	Amount injected per myotube	Injected protein as percentage of myotube protein
Haemoglobin	29 pg	1.0	20 pg	0.66
RNAase A	15 fg	1.5	22 fg	0.0005
Bovine serum albumin	7.0 fg	15.0	105 fg	0.0024

Fig. 1. *Fluorescent localization of haemoglobin in myotubes*

Erythrocyte haemoglobin was microinjected into myotube cultures and the cultures were fixed and incubated with fluorescein isothiocyanate-conjugated antibodies to human haemoglobin as described in the text. A, C and E, phase-contrast field of myotube cultures; B, D and F, fluorescent image of myotubes. The bar in F represents 10 μ m.

cyte membrane. Our average percentage capture of labelled protein by ghosts in these experiments was 7% for bovine serum albumin and 15% for RNAase A. The loading efficiency for both proteins was independent of protein concentration ranging from 0.001 to 10.0 mg/ml. If membrane binding occurred, the apparent loading efficiency would increase at low protein concentrations. (4) Acid-soluble degradation products of proteins after microinjection are largely (>95%) moniodotyrosine, a compound that is not subject to re-utilization by the protein-synthetic machinery of the cell.

Our more recent studies indicate that microinjection of RNAase A or bovine serum albumin has no discernable effects on myotube morphology as examined by phase-contrast microscopy. Also, no phagocytosis of ghosts loaded with fluorescein-

labelled RNAase A was evident by fluorescent microscopy. Furthermore, microscopic examination of cultures after microinjection with fluoresceinated dextran revealed that our procedure for washing cultures successfully removed non-fused ghosts. By counting the number of fluorescent ghosts remaining on the plate, we calculated that if all these ghosts were phagocytosed, they could account at most for 1.5% of total radioactivity per plate in a microinjection experiment.

We have attempted to estimate the number of ghost fusions per myotube, but the high density of plating combined with the branching and length of the myotubes made this extremely difficult. As a rough estimate we calculated that if each myoblast divided twice before fusion and each myotube was composed of 20 fused myoblasts, then, on the basis of the radioactivity introduced into the cells by

Fig. 2. Effects of microinjection and PEG treatment on rates of protein synthesis

Muscle cultures were microinjected with unlabelled bovine serum albumin or treated with PEG in the absence of erythrocytes. Protein synthesis was measured by determining the incorporation of [^3H]tyrosine into cellular protein for a 1 h period. Measurements were taken after microinjection or PEG treatment at (a) 5–7 h, (b) 23–26 h and (c) 44–48 h. 'PEG-treated 1 and 2' represents replicate experiments done on two different days. Each result represents the mean for six cultures $\pm$ 1 s.d.

microinjection and the radioactivity per loaded ghost, one to two ghosts containing RNAase A fused per myotube. Fusion ratios for bovine serum albumin were more variable and ranged between 10 and 20 ghosts per myotube. For unknown reasons, ghosts loaded with basic proteins such as RNAase A, lysozyme (Neff *et al.*, 1981), or histones (Schlegel & Rechsteiner, 1978) fuse more poorly to a variety of cell types.

Primary muscle cultures are not a homogeneous population of cells but contain some fibroblasts as well. Preplating cells and the use of cytosine arabinoside to kill dividing fibroblasts greatly reduce this fibroblast contamination so that less than 5% of total nuclei in mature myotube cultures are found in

Fig. 3. Effects of microinjection on the degradation of muscle-cell proteins

Long-lived proteins were labelled with [^{14}C]tyrosine for 2 days and short-lived proteins were labelled with [^3H]tyrosine for 2 h as described in the text. One group of cultures was then microinjected with ghosts containing non-radioactive RNAase A (O) and the other group was subjected only to washes (●). Immediately after microinjection, rates of protein degradation were monitored in MEM containing horse serum, CEE and 2 mM-unlabelled tyrosine. Each point represents the average result for four cultures; error ($\pm$ 1 s.d.) is contained within the datum point.

non-myotube cells (McElligott *et al.*, 1981; Pezementi & Schmidt, 1981). The amount of radioactive protein microinjected into cultures of pure chicken fibroblasts (derived from primary muscle cultures) using the protocol for myotube microinjection indicates that fusion efficiencies are equivalent for fibroblasts and myotubes. Thus the fibroblasts can contribute no more than 5% to total degradation measurements.

Observation of microinjection

To visually document microinjection, we monitored the transfer of haemoglobin from erythrocytes to myotubes by staining with a fluorescent antibody to human haemoglobin. Owing to the large size of the myotube, not enough fluoresceinated bovine serum albumin or RNAase A could be microinjected into myotubes to be microscopically detectable. However, the amount of haemoglobin in erythrocytes far exceeds the amount of protein we are able to load into ghosts (Table 1). Hendil (1980) has shown that human erythrocytes can be fused with hamster fibroblasts and that the microinjected haemoglobin is distributed throughout the cytoplasm and excluded from nuclei. We observe a

similar pattern of haemoglobin distribution in myotubes (Fig. 1). We noted that not all myotubes in a culture are microinjected (Fig. 1), but scoring more than 100 fields indicated that 30–50% have received the haemoglobin. Whether certain myotubes are actually resistant to microinjection or whether intact erythrocytes simply fuse more poorly than loaded ghosts remains to be established.

Effects of microinjection on cellular metabolism

The microinjection procedure could conceivably disturb cellular protein metabolism due to exposure to PEG, integration of erythrocyte membrane into plasma membrane, or the presence of microinjected protein in the cytoplasm. We determined rates of protein synthesis by measuring the incorporation of [³H]tyrosine into cellular protein for a 1 h period at various times after the microinjection of unlabelled bovine serum albumin. Radioactive tyrosine was used for protein-synthesis measurements since this amino acid is not metabolized by muscle cells (Fulks *et al.*, 1975; Li & Goldberg, 1976). Furthermore, incorporation of radioactive tyrosine has been shown to accurately reflect rates of protein synthesis when high levels of unlabelled tyrosine (2 mM) are included in the media to flood intracellular pools (Fulks *et al.*, 1975; Li & Goldberg, 1976). Measurements at time points during the 2 days after microinjection (Fig. 2) indicated that there was slight, if any, inhibition of protein synthesis due to microinjection or PEG treatment.

The amounts of cellular protein for microinjected and PEG-treated plates as determined by the Lowry procedure are within 75–93% of controls, indicating some loss of protein. Possibly some cells are rinsed off the plate after PEG treatment, but the remaining cells appear to be normally functioning as judged by protein-synthesis and -degradation measurements.

To assess the effect of microinjection on the degradative machinery of the cell, the rates of degradation of endogenously labelled proteins were measured after microinjection of unlabelled RNAase A. The rates of degradation of short-lived proteins (labelled for 2 h) and long-lived proteins (labelled for 2 days) are not affected by the microinjection procedure at any time up to 28 h after microinjection (Fig. 3). Furthermore, Fig. 4 demonstrates that there is no relationship between fusion ratio and the half-life of microinjected bovine serum albumin, suggesting that the incorporation of erythrocyte membrane into myotube membrane does not affect the degradative mechanisms of the cells. These results indicate that microinjection has negligible effects on protein metabolism in chick muscle cells.

Degradation of microinjected proteins

Microinjected proteins appear to be catabolized by the same mechanisms responsible for the

Fig. 4. Effects of fusion ratio on the half-life ($t_{1/2}$) of microinjected bovine serum albumin

¹²⁵I-labelled bovine serum albumin was microinjected into muscle cultures and the degradative rate measured. The number of ghosts fused per myotube was calculated as described in the text. Half-lives were calculated by using linear regression analysis for five degradation time points taken for each plate, and each datum point represents a single plate. There was no significant correlation between the number of ghosts fused per myotube and the half-life of microinjected bovine serum albumin. The correlation coefficient ($\bar{r}$) was 0.37 ($P > 0.1$).

breakdown of endogenous cytosolic proteins, since cytosolic proteins microinjected into fibroblasts are degraded according to several of the known characteristics of intracellular protein degradation (Neff *et al.*, 1981). We chose to microinject RNAase A (M_r 13 700, pI 9.4) and bovine serum albumin (M_r 67 000, pI 4.8) into muscle cells for the following reasons. (1) These proteins have different physical properties; (2) The structure of RNAase A remains intact after iodination, whereas the structure of bovine serum albumin is altered. (3) After microinjection into fibroblasts, the half-life of RNAase A is longer than that of bovine serum albumin (Neff *et al.*, 1981). (4) The degradation of RNAase A is increased during deprivation of serum and hormones (Backer *et al.*, 1983), whereas the catabolism of bovine serum albumin is not affected (Neff *et al.*, 1981). (5) We expected the degradation of these microinjected proteins to be similar in chick myotubes, since several other characteristics of intracellular protein degradation are shared by cell types as diverse as hepatocytes, muscle cells and fibroblasts (Dice *et al.*, 1978; Vandenburgh & Kaufman, 1980; Ballard & Gunn, 1982; Janeczko & Etlinger, 1982; Mortimore, 1982; Auteri *et al.*, 1983). Such similarities include average degradative rates of cellular proteins, correlations between rates

Fig. 5. Effect of horse serum and chick-embryo extract on the degradation of microinjected (a) RNAase A and (b) bovine serum albumin

^{125}I -labelled RNAase A and bovine serum albumin was microinjected into muscle cells and the degradation of the proteins monitored in the presence (●) or absence (○) of horse serum and CEE. Each point represents the average result (± 1 S.D.) for seven cultures for bovine serum albumin and five cultures for RNAase A. A total of 1452 ± 376 d.p.m. of ^{125}I -labelled bovine serum albumin and 2234 ± 398 d.p.m. of ^{125}I -labelled RNAase A were microinjected per culture. Similar results were obtained in five separate experiments for RNAase A and two separate experiments for bovine serum albumin.

of degradation and protein size, charge and/or presence of a carbohydrate moiety, and regulation of degradation by serum, growth factors and hormones.

The half-life of RNAase A microinjected into myotubes is 40h in the presence of serum and CEE, whereas the half-life of bovine serum albumin is 17h (Fig. 5). The half-life of RNAase A microinjected into human fibroblasts is somewhat variable, but 40h is within the observed range (Neff *et al.*, 1981). The half-life of microinjected bovine serum albumin is approx. 20h in human fibroblasts (Neff *et al.*, 1981) and several other cell types (Zavortink *et al.*, 1979).

Serum and CEE withdrawal increase the degradative rate of microinjected RNAase A by 60%, but do not affect the catabolism of bovine serum albumin (Fig. 5). This result demonstrates that the enhanced proteolysis in myotubes can distinguish

among different proteins. Whether the inability of myotubes to regulate catabolism of bovine serum albumin is related to the altered structure of this protein after iodination (Neff *et al.*, 1981) remains to be established. In any event, it is well-known that serum withdrawal enhances the average degradative rate of cellular proteins in myotubes as well as other cell types and that the enhanced catabolism can be selective in that it applies to long-lived proteins only (Vandenburgh & Kaufman, 1980; Ballard & Gunn, 1982; Mortimore, 1982; Janeczko & Etlinger, 1982; Auteri *et al.*, 1983). Thus the degradation of microinjected proteins in chick myotube cultures appears to be similar to degradation reported for other cell types. It may now be possible to use microinjection to analyse the degradative pathways and regulation of protein catabolism in muscle cells in detail.

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