Activity of lysosomal cysteine proteinase during differentiation of rat skeletal muscle

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Cysteine-proteinase activities were measured in extracts of pre- and post-fusion populations of rat myogenic line L₆ cells and in extracts of whole rat muscle. Activities of cathepsins B, L and H were compared. The substrates used included Z-Phe-Arg-NMec (cathepsins B and L), Z-Arg-Arg-NMec (cathepsin B), and Arg-NMec (cathepsin H) (where Z = benzyloxycarbonyl, and NMec = 4-methyl-7-coumarylamide); the enzyme activities were more specifically differentiated by appropriate concentrations of the inhibitors Z-Phe-Phe-CHN₂ (CHN₂ = diazomethane), bestatin and E-64 [L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane]. These experiments have demonstrated the feasibility of determining the cysteine-proteinase activities of myoblasts from a single (60mm-diameter) Petri dish, with enzyme concentrations in the range of 5–20 ng/ml. Specific activities of the enzymes in L₆ cells increased 2–20-fold after fusion. Concentrations of cysteine proteinases in extracts from cultured myoblasts were two orders of magnitude greater than those in muscle-tissue extracts. Cultured-cell extracts contained endogenous inhibitor(s) to purified rat cathepsins B, L and H.

Rat muscle homogenate has been shown to contain relatively low proteolytic activity (Bird *et al.*, 1968; Bird, 1975), which is in accordance with the low average turnover of muscle proteins (Bohley *et al.*, 1980). It was questionable as to whether the predominantly lysosomal proteolytic activity of muscle homogenate originated from the lysosomes of myocytes or various other cell types found in these highly heterogeneous tissues. Accordingly, we have focused our attention on the proteolytic activity of lysosomal enzymes from muscle cells differentiating *in vitro* (Bird *et al.*, 1981; St. John *et al.*, 1981; McElligott *et al.*, 1981). The homogeneous cell populations provided by cultures of established rat myogenic lines avoid the uncertainty that enzyme

activities originate from non-muscle cells, neural fragments or Schwann cells. These cultures, furthermore, allow growth under defined conditions to provide pure populations representative of several distinct morphological stages with various degrees of myogenic differentiation: pre-fusion (myoblasts), post-fusion (myotubes) and a non-fusing subclone that provided confluent but un-fused cultures.

Our initial studies have demonstrated the existence of a dynamic lysosomal apparatus with a full complement of acid hydrolases in the cultured muscle cells. Vital staining with Acridine Orange, a metachromatic dye used as a fluorescent marker for lysosomes, revealed prominent orange staining in numerous discrete vesicles randomly dispersed in the perinuclear regions of the cells. Electron microscopy of the cells demonstrated a reaction product for the lysosomal marker acid phosphatase in the same vesicles that stained for Acridine Orange. Our initial studies of the cysteine proteinases, with azocasein as substrate and incubation at pH 6.0, suggested that the specific activities of the enzymes were at least two orders of magnitude higher in cultured cells as compared with muscle homogenates, and that some of the enzyme activities increased significantly after fusion of the myoblasts.

In the experiments described below we have

Abbreviations used: Z, benzyloxycarbonyl; NMec, 4-methyl-7-coumarylamide; CHN₂, diazomethane; E-64, L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane; NNap, 2-naphthylamide; Bz, benzoyl.

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studied in detail the concentrations of cathepsins B, H and L in cultured myoblasts, very sensitive fluorescent substrates and inhibitors being used to check their suitability to serve as specific substrates for each of the cysteine proteinases in crude cell extracts. The activities in the cell extracts were compared with those of whole muscle extracts from rats and with purified enzymes that were standardized by active-site titration with E-64. We also have demonstrated the presence of endogenous inhibitor(s) to the cysteine proteinases in the cultured muscle cells.

Materials and methods

Materials

Male Sprague-Dawley rats (1 month old) were purchased from Charles River (Wilmington, MA, U.S.A.). Triton X-100 was purchased from J. T. Baker, Phillipsburg, NJ, U.S.A., and Brij 35 from Fisher Chemicals, Fairlawn, NJ, U.S.A. The two detergents were purified before use (Ashani & Catravas, 1980). Z-Phe-Phe-CHN₂ was synthesized and kindly provided by Dr. E. N. Shaw, Brookhaven National Laboratory, Upton, NY, U.S.A. Bestatin was kindly given by Dr. J. K. McDonald, Medical University of South Carolina, Charleston, SC, U.S.A. E-64 was purchased from the Peptide Institute, Osaka, Japan. Puromycin was purchased from Sigma Chemicals, St. Louis, MO, U.S.A. Z-Phe-Arg-NMec and Z-Arg-Arg-NMec were purchased from Bachem Chemicals, Torrance, CA, U.S.A.; Arg-NMec and 7-amino-4-methylcoumarin were purchased from Enzyme Systems Products, Livermore, CA, U.S.A.

Muscle extracts

Rats were anaesthetized with diethyl ether and killed by decapitation. Thigh and gastrocnemius muscles were removed, trimmed of most nervous and connective tissues, minced and homogenized in a VirTis homogenizer as a 20% (w/v) homogenate in 100 mM-acetate buffer (pH 5.5)/0.2% Triton X-100 at 4°C. The homogenate was centrifuged at 32000g for 15 min and the supernatant retained. Part of the supernatant was made 1 mM with respect to Na₂EDTA and was stored at -20°C in 1 ml portions which were thawed only once; this extract was used to determine cysteine-proteinase activities. The remaining extract without EDTA was not frozen and it was used for measurement of aminopeptidase activity. The extracts were diluted with 0.1% Brij 35 immediately before use to give a concentration of about 8 mg of protein/ml.

Cell culture

Cells of the rat muscle-cell line L₆ were cultured and treated before lysis as described by Bird *et al.*

(1981). The pre-fusion cells were plated at a sufficiently low density to prevent fusion during the culture period of 5 days. The post-fusion cells were maintained for 14 days *in vitro*, at which time they were confluent and fused into multinucleated straps. The washed cultured cells were then treated as described above for the minced tissue. The extracts were diluted with 0.1% Brij 35 before use to give a protein concentration of the myoblast extract of about 60 µg/ml and of the myotube extract of about 20 µg/ml.

Purified enzymes from rat liver

Purifications were as described for cathepsin B (EC 3.4.22.1) (Kirschke *et al.*, 1976), cathepsin L (EC 3.4.22.15) (Kirschke *et al.*, 1977a, 1982), and cathepsin H (EC 3.4.22.16) (Kirschke *et al.*, 1977b). The enzymes were stored in 100 mM-acetate buffer (pH 5.0)/0.5 mM-HgCl₂/1 mM-Na₂EDTA. The catalytically active concentrations of cathepsins B and L were titrated with E-64 (Barrett & Kirschke, 1981; Barrett *et al.*, 1982). The specific activity of cathepsin H was based on the protein content rather than the catalytically active concentration of the enzyme.

Enzyme assays

All enzyme activities were determined with a general procedure utilizing methylcoumarylamide substrates (Barrett, 1980). A portion (50 µl) of enzyme solution in 0.1% Brij 35 was mixed with 200 µl of activated buffer, and 50 µl of inhibitor solution or 0.1% Brij 35 was added. After exactly 5 min at 40°C, 200 µl of substrate solution was added to the preincubation mixture. The incubation was stopped after 10 min at 40°C by addition of 2.0 ml of 100 mM-monochloroacetic acid in 100 mM-acetate buffer, pH 4.3. Fluorescence was measured in an Aminco-Bowman spectrophotofluorimeter with an excitation wavelength set to 360 nm and emission wavelength set to 460 nm. Zero was set with 80 mM-chloroacetic acid in acetate buffer and 100 was set with 0.1 µM-aminomethylcoumarin in the same buffer. A ΔF of 100 represents 25 µ-units of enzyme in the tube in a 10 min assay (1 unit is the release of 1 µmol of product/min).

All methylcoumarylamide substrates were stored as 10 mM-solutions in dimethyl sulphoxide at 4°C and diluted with water to the appropriate concentration before use.

Cathepsin B. The substrate, Z-Arg-Arg-NMec, was 12.5 µM in water (5 µM in the assay). The incubation buffer was 250 mM-phosphate buffer, pH 6.0, containing 2.5 mM-Na₂EDTA and 5 mM-dithiothreitol (added freshly).

Cathepsin L. The substrate, Z-Phe-Arg-NMec, was 12.5 µM in water (5 µM in the assay). The incubation buffer was 250 mM-sodium acetate buffer, pH 5.5,

containing 2.5 mM- Na_2EDTA and 5 mM-dithiothreitol (added freshly).

Cathepsin H. The substrate, Arg-NMec, was 50 μM in water (20 μM in the assay). The incubation buffer was 250 mM-phosphate buffer, pH 6.8, containing 2.5 mM- Na_2EDTA and 5 mM-dithiothreitol (added freshly).

Arginine aminopeptidase

The substrate, Arg-NMec, was 50 μM in water (20 μM in the assay). The incubation buffer was 200 mM-phosphate buffer, pH 6.8, containing 300 mM-NaCl.

Synthetic inhibitors

All inhibitors were 10 mM in dimethyl sulphoxide during storage at 4°C. They were diluted with 0.1% Brij 35 in water before use, to the following concentrations: Z-Phe-Phe- CHN_2 , 3 μM ; E-64, 0.6 mM and 10 μM ; bestatin, 100 μM ; puromycin, 0.6 mM.

Endogenous inhibitors

Portions (300 μl) of the extracts of muscle cells were adjusted to pH 9–10 by addition of 100 mM-NaOH. After 60 min at 40°C, the extracts were re-adjusted to pH 5.5 by addition of 100 mM-HCl

and acetate buffer to give a volume of 600 μl . The extracts did not show cysteine-proteinase activity after the alkalination treatment. The preincubation time of the purified enzymes and inhibitors without substrate was 5 min at 40°C.

Determination of protein. Protein in the muscle extracts was precipitated in 10% (w/v) trichloroacetic acid at 4°C. The pellet was dissolved in 1 M-NaOH and 'neutralized' (pH 5–10) with 1 M-HCl. The protein concentrations were then measured by a modified Lowry method (Langner *et al.*, 1971) and with fluorescamine (Böhlen *et al.*, 1973), with bovine serum albumin as standard. The trichloroacetic acid-precipitable protein was used for the protein determinations because it was found that about 25% of the Lowry-positive compounds in the cultured cells were not precipitable, as opposed to only 6–8% of the compounds from whole tissue extracts.

Results and discussion

Substrate and inhibitors

The combined use of highly sensitive substrates and inhibitors to distinguish between several lysosomal cysteine proteinases are summarized in Tables 1 and 2. Cathepsin B was determined with Z-Arg-Arg-NMec as substrate. Both Z-Arg-Arg-

Table 1. Cysteine-proteinase substrates

Assay substrate	Lysosomal cysteine proteinase ...	Activity (+) or no activity (–) shown towards substrate by:		
		Cathepsin B	Cathepsin H	Cathepsin L
Bz-Arg-NNap		+	+	–
Z-Arg-Arg-NNap		+	–	–
Arg-NNap		–	+	–
Azocasein		+	+	+
Z-Arg-Arg-NMec		+	–	–
Arg-NMec		–	+	–
Z-Phe-Arg-NMec		+	–	+
Z-Phe-Arg-NMec + Z-Phe-Phe- CHN_2 *		+	–	–

* Only valid for the enzymes from rat; inhibitor must be used in a specific concentration.

Table 2. Specific activities of cysteine proteinases purified from rat liver lysosomes

Enzyme	Substrate	Specific activity (units/mg of protein)	Inhibitor	Concn. (μM)	Inhibition (%)
Cathepsin B	Z-Arg-Arg-NMec	13.90			
	Z-Phe-Arg-NMec	12.60			
			Z-Phe-Phe- CHN_2	0.5	1–2
Cathepsin H	Arg-NMec	3.50	E-64	2	100
			E-64	100	100
			E-64	2	85–90
			Bestatin	20	0
Cathepsin L	Z-Phe-Arg-NMec	22.00			
	Z-Arg-Arg-NMec	0.05			
			Z-Phe-Phe- CHN_2	0.5	100
			E-64	2	100

NNap (McDonald & Ellis, 1975) and Z-Arg-Arg-NMec (Barrett & Kirschke, 1981) are highly specific substrates for cathepsin B. The only other enzyme that has been found to hydrolyse Z-Arg-Arg-NMec was a proteinase similar to cathepsin B from tumour cells (Mort *et al.*, 1980). The methylcoumarylamide substrates have been shown to be better substrates than the corresponding naphthylamides, as illustrated for cathepsin B by a greater k_{cat}/K_m for Z-Arg-Arg-NMec ($3419 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) than for Z-Arg-Arg-NNap ($887 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) (Barrett & Kirschke, 1981). The methylcoumarylamide substrates are also superior by reason of their very high sensitivity (Barrett, 1980).

In the present study cathepsin L was determined with Z-Phe-Arg-NMec as substrate and Z-Phe-Phe-CHN₂ in a definite concentration as a specific inhibitor of the enzyme from rat. No specific substrate has yet been found for cathepsin L. Proteins have been used as substrates for the determination of this lysosomal enzyme: azocasein with urea, pH 5.0, or ¹⁴C- and ³H-labelled cytosol proteins, pH 3.0 (Riemann *et al.*, 1982). Z-Phe-Arg-NMec is superior to these substrates because of its high sensitivity (about 10 ng of enzyme/ml can be determined). Z-Phe-Arg-NMec is rapidly hydrolysed by cathepsin B (Barrett, 1980) and cathepsin L (Kirschke & Shaw, 1981; Barrett & Kirschke, 1981). It is also hydrolysed by plasma kallikrein, a serine proteinase which cannot be inhibited by E-64 (Barrett *et al.*, 1982). It is not known whether other intracellular enzymes may also hydrolyse this substrate. The activities of cathepsin B and cathepsin L from rat towards Z-Phe-Arg-NMec may be distinguished by judicious use of the inhibitor Z-Phe-Phe-CHN₂. This substance reacts irreversibly and rapidly with cathepsin L from rat, and mainly reversibly with cathepsin B from rat and human liver, though irreversibly with cathepsin B from bovine spleen (Kirschke & Shaw, 1981). Therefore the reaction of Z-Phe-Phe-CHN₂ as a specific inhibitor of rat cathepsin L is very much dependent on the conditions of the assay. We found that purified cathepsin L from rat liver in the appropriate concentration was inhibited 100% after a preincubation of 5 min with 0.5 μM -Z-Phe-Phe-CHN₂ in the preincubation mixture (Table 2). It was necessary to check the inhibition of cathepsin B under the same conditions, except that Z-Arg-Arg-NMec was used as its specific substrate. (The rate of hydrolysis by cathepsin B of Z-Arg-Arg-NMec and Z-Phe-Arg-NMec at the low concentrations used in these assays is nearly the same.) Z-Phe-Phe-CHN₂ showed negligible (2% or less) inhibition of both purified cathepsin B from rat liver and cathepsin B in the crude muscle extracts (Table 2 and Table 4 below).

Cathepsin H was determined with Arg-NMec as

substrate and E-64 as inhibitor. Arg-NMec and Arg-NNap are good substrates for cathepsin H (Barrett, 1980) and arginine aminopeptidase (McDonald & Barrett, 1983), but one should bear in mind that the substrate is also hydrolysed by soluble alanine aminopeptidase and leucine aminopeptidase (McDonald & Barrett, 1983). Bestatin has no influence on the activity of cathepsin H (Table 2), but inhibits arginine aminopeptidase competitively ($K_i = 1.4 \times 10^{-8} \text{ M}$) (Suda *et al.*, 1976) and leucine aminopeptidase (McDonald & Barrett, 1983), whereas puromycin will inhibit alanine aminopeptidase (Kawata *et al.*, 1980). Na₂EDTA is inhibitory to arginine, leucine, and alanine aminopeptidases, but if these aminopeptidases are present in high enough concentrations in comparison with cathepsin H, their remaining activities would interfere with measuring the activity of cathepsin H. Cathepsin H can be inhibited by E-64. We have confirmed the finding of Barrett *et al.* (1982) that concentrations of E-64 which completely inhibit cathepsin H have no inhibitory effect on arginine aminopeptidase. Cathepsin H purified from rat liver was completely inhibited by 100 μM -E-64 under our assay conditions. If the concentration was lowered to 2 μM , inhibition was 85–90% (Table 2). The lower concentration would completely inhibit cathepsin H if the preincubation time was longer than 5 min (results not shown).

The use of the methylcoumarylamide substrates permits the determination of cathepsin L, cathepsin B and cathepsin H in solutions containing the enzymes in concentrations of 5–20 ng/ml. It is now possible to measure these enzymes in an extract of cells in culture from a single 60 mm-diameter Petri dish. One plate usually contains an average of 18 μg of protein from pre-fusion cells or 160 μg from post-fusion cells.

There are some uncertainties that could restrict the application of the methods used in the present study. We do not yet have exhaustive knowledge of the intracellular enzymes, their substrate specificity and their reactivity with synthesis inhibitors. It is not known whether the substrate specificity of lysosomal cysteine proteinases of different organs is the same within one species. Some evidence has been found of species differences in the lysosomal cysteine proteinases concerning their reaction with synthetic inhibitors (Kirschke & Shaw, 1981).

Enzyme activities in tissues and cells

The preceding experiments demonstrate that it is possible to measure and differentiate the specific lysosomal cysteine proteinases in cell extracts of rat tissue and cell homogenates. The strategy is to measure (a) cathepsin B with Z-Arg-Arg-NMec as substrate (the activity can be inhibited by E-64), (b) cathepsin L with Z-Phe-Arg-NMec as substrate and

Z-Phe-Phe-CHN₂ in a definite concentration as specific inhibitor of this enzyme from rat, and (c) cathepsin H with Arg-NMec as substrate and E-64 as inhibitor of cathepsin H.

Our previous studies demonstrated very low specific activities of the cysteine proteinases towards the 2-naphthylamide substrates in cell extracts of rat muscle (Bird *et al.*, 1981). The present study demonstrated that the fluorescent coumarylamide substrates were approx. 20 times more sensitive than the 2-naphthylamide substrates for the cysteine proteinases in muscle tissue extracts (Table 3), and that the extracts of cultured myoblasts contained concentrations of the cysteine proteinases at least two orders of magnitude greater than the muscle tissue extracts (Tables 3 and 4; different units were used to facilitate comparison). It was noteworthy that the increase in specific activity in the extracts of myoblasts, in comparison with extracts of whole tissue, was different for these lysosomal proteinases. The specific activity of cathepsin L was 513 times, cathepsin B was 133 times and cathepsin H was 13 times higher in myoblasts than in muscle extracts

(Table 3). The reason may have been that different cell types of the muscle tissue contained different amounts of cystine proteinases in their lysosomes; they also may have had different concentrations of endogenous inhibitors, which could exhibit different actions on the different cysteine proteinases. We have demonstrated the presence of a potent endogenous inhibitor(s) to these enzymes in muscle tissue as well as in cultured muscle cells (see below).

During differentiation, the mononucleated spindle-shaped embryonic myoblasts proliferate for 3–4 days before they begin to fuse and differentiate into multinucleated myotubes. These fibres exhibit typical cross-striations and occasionally contract spontaneously in the culture dishes. Bird *et al.* (1981) demonstrated the presence of lysosomes in rat myoblasts, myotubes and in a fusion-negative mutant clone, and further showed an increase of certain lysosomal enzyme activities during differentiation. Cathepsin D activity remained unchanged, but the cystine-proteinase specific activities were increased 5–7-fold after fusion. We have confirmed this finding in the present study and can specify an increase in the activities of cathepsin L (about 5-fold), cathepsin B (about 2-fold) and cathepsin H (about 7-fold) (Table 4). This result was surprising considering that these enzymes were located in the same kind of lysosomes.

The concentrations of several proteins, mainly muscle-specific proteins, have been shown to increase after fusion of myoblasts [for review, see Buckingham (1977)]. It has been found that the morphological differentiation does not necessarily enhance the synthesis of all proteins. Fusion-negative mutants and myoblasts treated with differentiation-inhibiting agents (e.g. 5-bromodeoxyuridine) were able to rapidly produce some proteins after a culture period in which they normally underwent fusion. In view of the increase in protein concentrations after fusion, one could assume that the increase in the cysteine proteinases was especially

Table 3. Comparison of specific activities in rat muscle tissue and cultured muscle cells (*L₆* clone)

Assay conditions: cell extracts were preincubated for 5 min at 40°C in the presence of dithiothreitol (3.3 mM), 1.7 mM-EDTA and inhibitors (pH 5.5–6.8). Incubation was for 10 min at 40°C. Substrate concentrations of 5–20 µM were used. Results are means ± s.d. (*n* = 5).

Enzymes	Specific activity (µ-units/mg of protein)		
	Muscle tissue	Myoblasts (pre-fusion)	Myotubes (post-fusion)
Cathepsin B	46 ± 6	6100 ± 1300	14 000 ± 3000
L	6 ± 2	3080 ± 610	16 260 ± 2900
H	99 ± 15	1280 ± 380	9200 ± 1200

Table 4. Specific activities during differentiation (myogenic cells of *L₆* clone)

For details of the assay conditions, see the legend to Table 3.

Enzyme	Substrate	Inhibitor (concn.)	Myoblasts (pre-fusion)		Myotubes (post-fusion)		Increase in activity (fold)
			Sp. activity (m-units/mg)	Inhibition (%)	Sp. activity (m-units/mg)	Inhibition (%)	
Cathepsin B	Z-Arg-Arg-NMec		6.10 ± 1.3		14.10 ± 3.0		2.3
		E-64 (2 µM)	0	100	0	100	
		Z-Phe-Phe-CHN ₂ (0.5 µM)	6.10	0	14.00	0	
Cathepsin L	Z-Phe-Arg-NMec		8.70 ± 1.9		32.50 ± 5.3		5.3
		E-64 (2 µM)	0	100	0	100	
		Z-Phe-Phe-CHN ₂ (0.5 µM)	5.62	35	16.24	50	
		Z-Phe-Phe-CHN ₂ -inhibited activity	3.08		16.26		
Cathepsin H	Arg-NMec		1.28 ± 0.4		10.03 ± 1.5		7.2
		E-64 (100 µM)	0	100	0.83	92	

high. By using the specific activities of purified enzymes from rat liver as the basis of our calculations (Table 2), and assuming that muscle enzymes and liver enzymes have the same specific activities, we estimated that the cysteine proteinases represented 0.02–0.05% of total protein before fusion and 0.07–0.3% after fusion. In the myotubes (after fusion), cathepsin L represented 0.07%, cathepsin B 0.10% and cathepsin H 0.26% of protein.

Endogenous inhibitors

Endogenous inhibitors of cysteine proteinases have been found in the cytoplasmic extract of several mammalian tissues [see Barrett (1981) for review]. We reported an endogenous inhibitor to cathepsin B in the cytoplasmic extract of muscle tissue (Bird & Schwartz, 1977; Schwartz & Bird, 1977) which has also been found in skeletal and heart muscles of the rat, cow, rabbit, pig and human (Bird *et al.*, 1978). This inhibitor has recently been purified from muscle tissue (Lenney *et al.*, 1979; Brooks & Bird, 1981) and found to be active against cathepsins B and H. Cathepsin L has not been tested previously with an inhibitor from muscle. It has been found that an inhibitor from human and rat epidermis reacted very quickly with cathepsins L and H and slowly with cathepsin B (Ohtani *et al.*, 1982). The presence of an endogenous inhibitor of the calcium-dependent proteinase II in myoblasts before fusion and its apparent destruction during differentiation has been discussed by Kaur & Sanwal (1981).

We determined the levels of inhibitors of cysteine proteinases in extracts of cultured muscle cells before as well as after fusion. We presumed that these inhibitors had the same properties that have been described for endogenous inhibitors from skin (Järvinen, 1978) and from egg white (Barrett, 1981), i.e., they are stable and become separated from enzymes when the latter are denatured by treatment at pH 9–10. The alkaline treatment of the extract was extended to 60 min at 40°C, after which no activity of the cysteine proteinases could be detected. Because there is no direct method to measure the inhibitor concentration in the presence of the enzyme, it is impossible to calculate the recovery of the endogenous inhibitor after the alkaline treatment, and we do not know whether the cells contained additional inhibitors that were unstable at pH 10. The extracts were diluted after alkaline treatment to approximately the same protein concentration as had been used to measure enzyme activity, and they were then incubated with purified cathepsins from rat liver lysosomes. Inhibition was expressed as a percentage change in specific activities of the enzymes (Table 5). These preliminary studies showed that cultured muscle cells before and after fusion contained inhibitors that could react under the experimental conditions with the enzymes

Table 5. *Endogenous inhibition of purified enzymes by cell extracts*

Cell extracts were treated with 100 mM-NaOH to destroy acid proteinases, then re-adjusted to pH 5.5. Extracts were allowed to react with purified rat liver enzymes. Cathepsin L and B activity was determined by titrating the catalytically active concentrations with E-64. The specific activity of cathepsin H was based on the protein content of enzyme. Percentage inhibitions were calculated for a concentration of 3 µg of cell-extract protein per assay.

Enzymes	Inhibition (%)	
	Pre-fusion	Post-fusion
Cathepsin L (14.0 µ-units)	41	54
Cathepsin B (19.0 µ-units)	17	16
Cathepsin H (12.3 µ-units)	13	41

and inhibited about 50% or less of the active cysteine proteinases in the given high dilution. The three cysteine proteinases tested were all partially inhibited by the cellular extract, but to a different extent. There was an apparent increase in the concentrations of inhibitors of cathepsins L and H after cell fusion. Of obvious importance would be the fact that we now have evidence that muscle cells (as opposed to other cell types in muscle tissue) contain the endogenous inhibitor(s).

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