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Calpeptin Blocks Myogenic Time-dependent Loss of Cytoskeletal Proteins and Membrane Fusion of Chick Embryonic Myoblasts

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The protein level of cytoskeletons in cultured myoblasts was found to gradually decrease during the course of myogenesis. This decrease, however, could be prevented by treating the cells with calpeptin (benzyloxycarbonyl-Leu-nLeu-H), a cell penetrating inhibitor of calpain. In contrast, E-64, which also is a potent inhibitor of calpain but can not be transported into the cells, showed little or no effect. In addition, the treatment of calpeptin was found to stabilize a number of specific cytoskeletal proteins from degradation but without any effect on the pattern of total cells proteins. Furthermore, calpeptin, but not E-64, blocked myoblast fusion in a dose-dependent manner. These results suggest that calpain is responsible for the myogenic time-dependent loss of cytoskeletal proteins and that the degradative process is associated with myoblast fusion. These results also suggest that the differential effects of the calpain inhibitors depend on the permeability of the drugs across the cell membrane.

KEY WORDS: Calpeptin, Calpain, Cytoskeletal proteins, Myoblast fusion

A prominent event in the differentiation of skeletal muscle cells is the fusion of mononucleated myoblasts into multinucleated myotubes (Bischoff and Holtzer, 1969; O'Neill and Stockdale, 1972). This process absolutely requires influx of Ca^{2+} (David *et al.*, 1981) and accompanies various cellular events including reorganization of cytoskeleton (Fulton *et al.*, 1981) and redistribution of membrane components (Pauw and David, 1979). Intracellular proteolysis has been suggested to involve in the myogenic cellular processes and therefore to be requisite for myoblast fusion (Wakelam, 1985). Calpain, which is an intracellular thiol-protease and absolutely requires Ca^{2+} for activity, has been suggested to play an important role(s) in myoblast fusion. It has been shown that calpain is relocalized from cytosol to membrane during the fusion and suggested that its intracellular redistribution is responsible for the cytoskeletal reorganization (Schollmeyer, 1986a, 1986b). In addition, both the activity and protein levels of calpain have recently been shown to increase dramatically during early period of myogenesis (Kwak *et al.*, 1993). Furthermore. calpain is capable of hydrolyzing *in vitro* a variety of cytoskeletal proteins, such as filamin, talin, vimentin, fodrin, neurofilaments and microtubule-

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associated proteins (Suzuki and Ohno, 1990). Therefore, calpain is a candidate protease that involves in myoblast fusion.

In an attempt to elucidate the role of calpain in myoblast fusion, we investigated the effects of calpain inhibitors on the rearrangement of cytoskeletal proteins during myogenesis. Calpeptin, benzyloxycarbonyl-Leu-nLeu-H, is a peptide aldehyde inhibitor of calpain and does not contain any charged group (Tsujinaka *et al.*, 1988). Therefore, this drug can effectively penetrate into cells unlike other inhibitors of calpain, such leupeptin and E-64 (Aoyagi and Umezawa, 1975; Hanada *et al.*, 1978), which have a positively charged guanidium group at neutral pH. In the present study, we demonstrate that the cell-penetrating inhibitor of calpain, but not the other inhibitors that can not come across the cell membrane, blocks the myogenic time-dependent loss of cytoskeletal proteins and thereby prevents the membrane fusion of embryonic myoblasts in culture.

Materials and Methods

Materials

Calpeptin was provided by Dr. J. Kambayashi (Osaka University, Japan). The 80 kDa catalytic subunit of calpain was purified to apparent homogeneity as described previously (Chung *et al.*, 1993). Culture agents were obtained from Gibco and E-64 and leupeptin were from Enzyme Product System (Tokyo, Japan). All other chemicals were purchased from Sigma.

Cell Culture

Myoblasts from breast muscle of 12-day-old chick embryos were prepared as described previously (Kwak, *et al.*, 1993; Kim *et al.*, 1992). The cells were plated on collagen-coated culture dishes at a concentration of 5×10^5 cells/ml in Eagle's essential medium containing 10% horse serum, 10% chick embryo extract and 1% antibiotic/antimycotic solution. One day after the cell seeding, the culture medium was changed with the same medium but containing 2% embryo extract. When needed, calpain inhibitors were treated to the culture medium at the time of medium change.

Preparation of Cytoskeletal Proteins

Myoblasts cultured for appropriate periods were harvested and washed 3 times with 10 mM Tris-HCl buffer (pH 7.8) containing 0.14 M NaCl. The cells were freeze-thawed once in a dry-ice/acetone bath and disrupted by 7 strokes of homogenization using a Teflon/Glass homogenizer. The resulting samples were spun for 10 sec in a microfuge to remove cell debris. Supernatants were collected, added with Triton X-100 to a final concentration of 0.5% (w/v), and incubated for 30 min at room temperature. After centrifugation of the samples for 3 min at 10,000 × g, pellets were washed twice with 10 mM Tris-HCl buffer (pH 7.8) containing 0.14 M NaCl and 0.5% Triton X-100 and resuspended in the same buffer by sonication. The suspensions were referred to as the cytoskeletal proteins.

Results and Discussion

A primary role of cytoskeletal proteins is to provide the framework of cell structure. Therefore, when cells are engaged in membrane fusion with adjacent cells, the cytoskeletal networks should be rendered prior to the cellular event. To examine if the myogenic fusion process accompanies the rearrangement of cytoskeleton, cytoskeletal proteins were isolated from the myoblasts cultured for various periods and assayed for their protein quantities. Fig. 1 shows that the total amounts of cytoskeletal proteins dramatically decrease during the course of myogenic differentiation. Because calpain is known to involve in the breakdown of cytoskeleton *in vitro* (Suzuki and Ohno, 1990), we tested if the treatment of calpain inhibitors can block the loss of cytoskeletal proteins. Calpeptin, a cell-penetrating inhibitor of calpain, almost completely prevented the loss of cytoskeletal proteins, indicating that the intracellular proteolysis by calpain is responsible for the loss of the proteins. However, E-64, which also inhibit calpain *in vitro* but can not penetrate into cells, showed little or no effect on the protein loss.

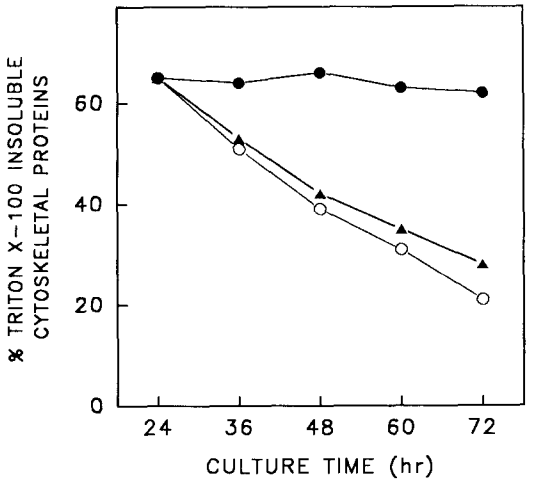

Fig. 1. Effects of calpain inhibitors on the changes in the level of cytoskeletal proteins during myogenesis. At the culture period of 24 h, myoblasts were treated with 0 (○), 100 μM calpeptin (●) or 100 μM E-64 (▲). After the treatment, the cells were further cultured and their cytoskeletal proteins were isolated at the indicated time as described in Materials and Methods.

Thus, it is likely that the differential effects of the calpain inhibitors on the breakdown of cytoskeletal proteins are due to their ability to be transported across the cell membrane.

Of interest is the finding that the increase in the loss of total cytoskeletal proteins is myogenic time-dependent. We have previously shown that the activity of calpain increases markedly as the myogenic differentiation proceeds (Kwak *et al.*, 1993). Furthermore, the protein level of calpain also increase in parallel with the increase in the calpain activity. On the other hand, the activity level of calpastatin, an endogeneous inhibitor of the protease, remains similar during entire period of myogenesis (Kwak *et al.*, 1993). Therefore, it seems clear that the increased loss of cytoskeletal proteins during the cource of myogenesis is due to the increase in overall capacity of Ca^{2+}-dependent proteolysis in differentiating myoblasts.

In order to determine whether the loss of cytoskeletal proteins is limited to certain, specific proteins or is resulted from the overall increase in the rate of proteolysis, myoblasts were cultured in the presence and absence of calpain inhibitors. From these cells, cytoskeletal proteins were isolated and subjected to gel electrophoretic analysis. As shown in Fig. 2B, treatment of calpeptin dramatically stabilized a number of proteins (indicated by arrows) while E-64 showed little or no effect. Leupeptin, which also is a peptide aldehyde inhibitor of calpain but contains a positive charged guanidium group, neither showed any effect (data not shown), supporting the notion that the permeability of the inhibitors is due for their differential effect. On the other hand, the gel electrophoretic pattern of total proteins remained very similar for both the cells cultured in the presence of calpeptin or E-64 and those cultured in its absence (Fig. 2A). These results clearly demonstrate that calpain is responsible for the massive breakdown of cytoskeletal proteins during myogenesis and yet this process is limited to a number of specific cytoskeletons.

To examine if the myogenic time-dependent loss of cytoskeletal proteins is indeed related with membrane fusion, myoblasts that had been cultured for 24 h were treated with calpeptin or E-

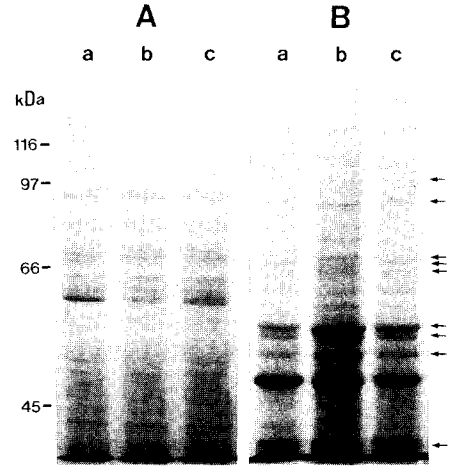

Fig. 2. Effects of calpain inhibitors on the hydrolysis of cytoskeletal proteins in cultured myoblasts. Myoblasts that had been cultured for 24 h were further cultured for the next 48 h in the absence (lane a) and presence of 100 μM calpeptin (b) or 100 μM E-64 (c). A. The cells were then harvested, disrupted by boiling in 2% sodium dodecyl sulfate (SDS), and subjected to polyacrylamide gel electrophoresis on 10% (w/v) slab gels in the presence of SDS (Laemmli, 1970). B. Cytoskeletal proteins were also isolated from the cultured cells and electrophoresed as above.

64. After the treatment, the cells were further cultured for the next 48 h and their ability to form myotubes were determined by the observation under a phase contrast microscope. As shown in Fig. 3, calpeptin, but not E-64, inhibited the membrane fusion in a dose-dependent manner. Thus, it is likely that the myogenic fusion process involves intracellular breakdown of cytoskeletal proteins, which is mediated by calpain, and hence inhibition of calpain can lead to the blockade of myoblast fusion.

It is noteworthy, however, that the specificity of calpeptin is not just restricted to calpain. This agent is also capable of inhibiting other thiol-proteases, such as cathepsin B (Tsujinaka *et al.*, 1988, Mehdi, 1991). Furthermore, lysosomotrophic amines, such as chloroquine and ammonium chloride, have been reported to interfere with myoblast fusion (Kent, 1982). However, it appears less likely that lysosomal proteases involve in cytoskeletal reorganization due to their subcelullar location. In addition, the calpain inhibitors do not contain any primary amine group that protonates at acidic pH for accumulation inside lysosomes, unlike lysosomotrophic agents (DeDuve *et al.*, 1974). Furthermore, cytoskeletal proteins, such as filamin, are much less susceptible to *in vitro* degradation by cathepsin B than calpain (Kwak *et al.*, 1993). Nevertheless, a possibility that other unknown, cytosolic thiol-protease(s) as well as lysosomal proteases including cathepsin B may be involved in myoblast fusion can not be totally excluded at present.

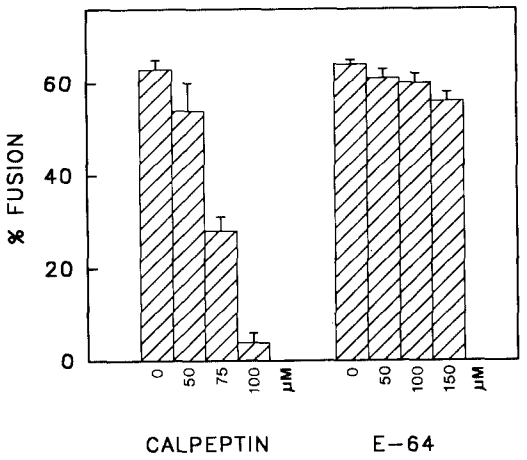

Fig. 3. Effects of calpain inhibitors on myoblast fusion. The cells were cultured as in Fig. 2 but in the presence of increasing concentrations of the drugs. Extent of myoblast fusion was expressed as the percent of the number of nuclei within fused cells to the total number of nuclei in ten independently chosed field under a phase contrast microscope. The cells containing more than 3 nuclei were regarded as the fused cells.

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계배 근원세포의 분화에 따른 세포 골격 단백질의 분해와 막 융합에 대한 ***Calpeptin***의 억제 효과

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배양 근원세포의 세포 골격 단백질의 양이 분화과정에 따라 점차 감소하는 것으로 나타났다. 이러한 세포 골격 단백질의 분해는, 세포막에 투과성을 나타내는 calpain의 저해제인 calpeptin(benzyloxycarbonyl-Leu-nLeu-H)의 처리에 의하여 억제될 수 있었다. 또한, calpeptin은 특정 세포 골격 단백질의 분해를 제한적으로 억제하였으나, 전체적인 세포 단백질의 양상에는 별 영향을 주지 않았다. 뿐만 아니라, calpeptin은 농도 의존적으로 근원세포의 융합을 억제하였다. 반면에, calpain의 강력한 저해제이지만 세포막에 투과성을 보이지 않는 E-64는 세포 골격 단백질의 분해와 막 융합에 아무런 효과를 나타내지 못하였다. 이러한 결과는 calpain이 근세포 분화 시기에 따라 세포 골격 단백질의 분해를 촉매하며, 이 분해 과정은 근원세포 융합에 필연적인 것으로 추측된다. 또한, 이 결과는 calpain 저해제들의 선별적 효과가 그들의 세포막에 대한 투과성에 기인함을 시사한다.