Therapeutic Effect of Camostat Mesilate on Duchenne Muscular Dystrophy in mdx Mice

Hitoshi Sawada, Kazumi Nagahiro, Yuhsuke Kikukawa, Susumu Ban, Reina Kakefuda, Tetsuo Shiomi, and Hideyoshi Yokosawa

Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060–812, Japan; and Sagashima Marine Biological Laboratory, Graduate School of Science, Nagoya University, Toba 517–0004, Japan. Received April 2, 2003; accepted April 21, 2003; published online May 2, 2003

Duchenne muscular dystrophy is known to be caused by a defective gene of dystrophin, a 427-kDa cytoskeletal protein, but the effective therapeutic drug is presently unavailable. We previously reported that a trypsin-like protease designated as dystrypsin is markedly activated in the muscle microsomal fraction immediately before onset of the clinical signs in mdx mice, a dystrophin-deficient hereditary animal model for human Duchenne muscular dystrophy. In order to examine the possible participation of dystrypsin in the occurrence of the disease, we investigated the therapeutic effects of dystrypsin inhibitors on the occurrence and progress of muscular dystrophy. Here, we show that camostat mesilate, a low-molecular-weight inhibitor of trypsin-like proteases, including dystrypsin, is a candidate drug for Duchenne muscular dystrophy.

Key words muscular dystrophy; trypsin-like enzyme; mdx mice; chemotherapy

Duchenne muscular dystrophy (DMD) is a degenerative disease with progressive muscle weakness and atrophy.1–3) One out of 3500 males is affected and dies at the age of about 20 years. DMD is an X-linked recessive disease caused by defective expression of dystrophin, a 427-kDa cytoskeletal protein with an α-actinin-like domain in the N-terminal side, a spectrin-like domain in the middle, and a Cys-rich domain in the C-terminal region.1–3) Despite the discovery of the primary gene defect, the molecular mechanisms triggering the onset of DMD are still unclear.4–6) Furthermore, an effective therapeutic drug for DMD has not yet been developed.

In order to elucidate the molecular mechanism of the onset of muscular dystrophy, it is essential to investigate the biochemical changes not in the affected muscles but in the muscles immediately before the onset of clinical signs of the disease, since a variety of secondary lesions would be elicited by the primary lesion. Although several proteinases, including calpain, cathepsin B/L and chymase have been reported to be activated during the progress of muscular dystrophy,7–10) little is known about the preclinical change in the muscle protease activity, which is potentially related to the manifestation of this disease.11)

We previously reported that a microsomal Boc-Val-Pro-Arg-MCA-hydrolyzing trypsin-like (or thrombin-like) protease, designated as dystrypsin, in hind-limb muscle is transiently activated immediately before the onset of the disease in the mdx mouse.12,13) a hereditary muscular dystrophic mouse with a defective dystrophin gene.6,14–16) However, it is presently unclear whether the apparent activation of dystrypsin is related to the manifestation of the disease. In the case that dystrypsin is involved in the occurrence of the disease via the limited proteolysis of muscular proteins, dystrypsin inhibitor is thought to be a candidate therapeutic drug for DMD.

In this context, we attempted to investigate the effects of potent inhibitors of dystrypsin on the occurrence or the progress of the disease. In our previous study, we found that gabexate mesilate is a potent inhibitor of dystrypsin.12) Since mesilate derivatives are currently used as therapeutic drugs for acute pancreatitis and DIC (disseminated intravascular coagulation), and also since these compounds are low-molecular-weight trypsin inhibitors with low immunogenicity, we attempted to examine the therapeutic effects of two mesilate derivatives, gabexate mesilate and camostat mesilate, on the manifestation of DMD by using mdx mice.

MATERIALS AND METHODS

Animals and Reagents The muscular dystrophic mouse C57BL/10-mdx and its control mouse C57BL/10-ScN were obtained from Clea Japan, Inc. Gabexate mesilate and camostat mesilate were generous gifts from Dr. K. Kato of Shira-tori Pharmaceutical Co., Ltd. and Drs. S. Suzuki and M. Kuri-mi of Torii Pharmaceutical Co., Ltd.

Assay for Enzyme and Inhibitor Activities Creatine kinase (CK) activity in plasma was determined using a commercially available kit (CPKII-HA TEST WAKO) according to the manufacturer’s protocol. One unit of CK activity was defined as the amount of enzyme that catalyzes the production of 1 μmol NADPH per min.

Dystrypsin was purified according to our previously described procedure.13) The purified enzyme (specific activity, 300 nmol/min/mg protein) was mixed with respective inhibitors, followed by incubation at 37 °C for 30 min in 0.45 ml of 50 mM Tris/HCl (pH 8.5). The residual enzymatic activity was determined by adding 50 μl of 100 μM Boc-Val-Pro-Arg-MCA (Peptide Institute, Osaka) as a substrate according to our previous procedure.13)

Administration Procedures and Evaluation of Therapeutic Effects Two hundred microliters of 1 mg/ml of gabexate mesilate and camostat mesilate, which had been dissolved in 5% glucose, was subcutaneously injected into the dorsal part of mdx mice aged more than 5 d twice a day for 30 d. As a control, 5% glucose was subcutaneously injected into mdx mice as described above. One of the above drugs and 5% glucose were injected into the same littermates (more than 6 newborn mice) after dividing them into two groups (3 or 4 individuals each) and marking them with colored ink. After administration, 70 μl of the blood was col-
lected with a heparin-coated capillary into a micortube containing 30 μl of 1000 unit/ml heparin dissolved in phosphate-buffered saline. The blood was centrifuged at 500×g for 10 min, and the resulting supernatant was used as a blood plasma preparation. Alternatively, 0.9% NaCl solution in the presence or absence of camostat mesilate was subcutaneously injected twice a day at a dose of 250 mg/kg/d essentially according to the abovementioned procedure. In this study, 7 and 6 littermates were treated with camostat mesilate and physiological saline, respectively, and the blood was collected at the age of 35 d. After collecting the blood, hind-limb skeletal muscles (gastrocnemius) of mdx mice were excised and fixed with 4% paraformaldehyde. After fixing, the specimens were subjected to hematoxylin-eosin staining for microscopic observation.

All the above experiments were carried out according to the Guide for the Care and Use of Laboratory Animals in Hokkaido University.

RESULTS AND DISCUSSION

Dytrypsin, which had been highly purified from 20-d-old hind-limb muscles of mdx mice essentially according to our previous procedure, was strongly inhibited by mesilate derivatives (for their structures, see Fig. 1), of which camostat mesilate showed the stronger inhibition (Fig. 2).

Next, in order to examine whether the mesilate derivatives are able to prevent the occurrence or the progression of DMD, we investigated the therapeutic effects of these compounds on DMD by successive administration in mdx mice aged 5 d, the timing of which corresponds to the stage before activation of dytrypsin in the muscles of mdx mice. The degree of the effect on DMD was judged by measuring the plasma CK activity in mdx mice, since CK in the affected muscles is known to leak to the blood stream.

The results after 1-month treatment are shown in Fig. 3. As shown in the figure, it was found that camostat mesilate, but not gabexate mesilate, is able to significantly lower the level of plasma CK activity. Although the plasma CK level in normal mice C57BL/10-ScN (less than 10%; data not shown) was much lower than that in camostat mesilate-treated mdx mice, it is evident that camostat mesilate is capable of preventing the leakage of muscle CK to the blood stream. The similar results were obtained in two series of the same experiments, although the plasma CK levels were more variable. When the administration dosage was modified depending on the body weight of each individual at each day, we also obtained a similar result that camostat mesilate is able to lower the plasma CK activity (see Fig. 3, Experiment 2).

In addition to the above biochemical studies, we also investigated the histological alterations in the hind-limb muscles of mdx mice. Although we could not detect the marked therapeutic effect by camostat mesilate, the treatment with camostat mesilate appears to be slightly effective on the basis of the number of small angulated muscular fibers, nucleus-centered regenerated fibers, and invading cells, which are typical symptoms in the muscles of mdx mice (Fig. 4). No obvious side effects of mesilate derivatives on blood coagulation in mdx mice were observed under the experimental conditions used in this study. All of the above-mentioned results lead us to propose that camostat mesilate is a candidate therapeutic drug for Duchenne muscular dystrophy, at least in mdx mice.

It has been reported that leupeptin (acetyl-Leu-Leu-Leu-H) showed a therapeutic effect on DMD in mdx mice and that the effect of leupeptin is thought to be due to the inhibitory
effect on calpain.\textsuperscript{17} Since leupeptin is not a specific inhibitor of calpain, it seems reasonable to conclude that a certain trypsin-like protease plays a key role in the occurrence or the progression of DMD in \textit{mdx} mice. Of course, dystrypsin and calpain are included in the candidate proteases involved in this process. Further detailed studies on the therapeutic effects of camostat mesilate and other trypsin inhibitors on DMD in \textit{mdx} mice and also in human patients are needed to open a new gate for chemotherapy of human DMD.

\textbf{Acknowledgements} This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Hokkaido Foundation for the Promotion of Scientific and Industrial Technology (Hokscitec), and Mochida Memorial Foundation for Medical and Pharmaceutical Research.

\section*{REFERENCES}