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Endocytotic internalization of α-2-macroglobulin: α-galactosidase Conjugate by cultured fibroblasts derived from Fabry hemizygote

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SUMMARY : Endocytotic internalization of α-galactosidase by cultured fibroblasts derived from a patient with Fabry's disease was achieved via receptor-mediated endocytosis of α-2-macroglobulin (α-2-M). α-galactosidase of coffee beans was conjugated to α-2-M when the latter was treated with trypsin. Internalization of the conjugate resulted in an increase of α-galactosidase activity in the crude cell extracts. The observed internalization was blocked by the presence of bacitracin, an inhibitor of binding between α-2-M and its receptor on the cell surface. When the cells were incubated at 4°C with the conjugate, internalization was also inhibited. The α-galactosidase activity in the cells was saturated when the concentration of the conjugate in the medium was 40 ug/ml. Since non-conjugated α-galactosidase was not effectively internalized, the observed internalization of the conjugate was mediated by recognition of α-2-M by its receptor. The effective internalization of α-galactosidase described in this paper has a potential use in the enzyme replacement therapy of Fabry's disease.

Fabry's disease is an x-chromosome liked disorder of glycosphingolipid metabolism originating from a deficiency of lysosomal hydrolase, α-galactosidase A, and results in an accumulation of certain glycosphingolipids(1). The enzyme replacement therapy is a possible treatment of such lysosomal storage diseases resulting from genetic mutation of lysosomal enzymes. But the progress of the therapy has been slow due to the difficulty of purification of human lysosomal enzymes and their general lability. While some non-human enzymes are

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commercially available, they have not been used for the therapy because no efficient method of their internalization into human cells has been devised and their antigenicity has been regarded as a potential harzard. As an attempt to alleviate some of these difficulties, we describe a potential application of human α-2-macroglobulin (α-2-M) to enzyme replacement therapy. When α-2-M forms a complex with proteinases, it undergoes a conformational change(2-5) and binds to α-2-M receptors on the cell surface(6). The complex is then internalized by the cell and delivered to the lysosome(7). During the reaction of α-2-M with proteinases, α-2-M can bind coexisting proteins other than proteinases(8). We found that α-galactosidase of coffee beans was conjugated to α-2-M when the latter was treated with trypsin. The α-2-M:α-galactosidase conjugate thus produced was internalized by cultured fibroblasts derived from a patient with Fabry's disease via receptor-mediated endocytosis of α-2-M. Internalization of the conjugate resulted in an increase of α-galactosidase activity in the cells.

MATERIAL AND METHODS

Preparation of α-2-M:α-galactosidase conjugate.

Human α-2-M was purified as described previously(9). α-galactosidase from coffee beans was purchased from Sigma Chem. Co.(St. Louis, MO). Trypsin (Sigma) was purified by benzamidine-sepharose 6B chromatography (Sigma)(10). 0.3 ml of α-galactosidase(4.7 mg/ml) dialyzed against 5 mM sodium phosphate buffer, pH 6.5 containing 150 mM NaCl was added to 0.3 ml of α-2-M(3mg/ml) in 20 mM phosphate buffer, pH 8.0, 150 mM NaCl. The mixture was then kept at 35°C, and 200 µl of purified trypsin(0.26 mg/ml)was added to the mixture. After 10 minutes' incubation, 40 µl of 5 mM N^{α}-p-tosyl -L-lysine chloromethyl ketone (TLCK) was added to the mixture and the activity of free as well as bound trypsin to α-2-M was blocked. The mixture was then dialyzed against 20 mM phosphate buffer, pH 5.8 containing 150 mM NaCl. After dialysis, the reaction mixture was applied to HPLC gel chromatography (TSK G 4000 SW) and then eluted with 50 mM phosphate buffer, pH 6.5 at a flow rate of 0.5 ml/min. The mixture of α-2-M:trypsin and α-2-M:α-galactosidase conjugates was completely separated from free α-galactosidase. Fractions containing the conjugate were collected for later experiments. α-galactosidase activity was assayed by a modified method of Beutler and Kuhl(11).

Time-dependency of uptake of the conjugate by GM2775 cells. Human fibroblasts derived from a patient with Fabry's disease (GM2775) was obtained from the Human Genetic Mutant Cell Repository (Camden, New Jersey, U.S.A.). The cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20 % fetal bovine serum and incubated at 37°C in an atmosphere of 10 % CO_2 and 90 % air. For the conjugate uptake experiments, the cells were grown to confluency in 35 mm dishes and the cell monolayers were rinsed twice with DMEM containing 5 mg/ml bovine serum albumin (DMEM-BSA), followed by incubation at 37°C or 4°C in this medium. After 15 min, the medium was replaced with new ones as follows : prewarmed DMEM-BSA containing 20 μg/ml conjugates at 37°C ; precooled DMEM-BSA containing 20 μg/ml conjugates at 4°C ; prewarmed DMEM-BSA containing 20 μg/ml conjugates and 1 mg/ml bacitracin at 37°C ; prewarmed DMEM-BSA containing 20 μg/ml trypsin-reacted α-2-M and 0.235 μg/ml α-galactosidase at 37°C. At the indicated time points, the medium was removed, and the cell monolayers were washed twice with DMEM and harvested with trypsin-EDTA. The cell suspension was centrifuged at 300xg for 5 min and the cell pellet was resuspended and washed twice with Dulbecco's phosphate buffered saline (PBS). The cells were then suspended in 0.25 ml of PBS and ruptured by three cycles of freeze-thawing. α-galactosidase activity in the crude cell extracts was then assayed. Specific activity of the enzyme was represented as μunits/mg of protein.

Dose-dependency of uptake of the conjugate by GM2775 cells. The cells (GM2775) were prepared as described in time-dependency study. After the cells were incubated at 37°C in DMEM-BSA for 15 min, the medium was replaced with DMEM-BSA containing α-2-M: α-galactosidase conjugates at various concentrations. The cells were incubated for 2 h and then harvested. And the activity in the crude cell extracts was assayed.

RESULTS AND DISCUSSION

Conjugate formation between α-2-M and α-galactosidase was effected by adding trypsin to a mixture of the former two proteins and the mixture of α-2-M and α-2-M: α-galactosidase conjugate was separated from free enzymes by HPLC gel chromatography on TSK G 4000 SW column (Data not shown). Fractions containing α-2-M exhibited α-galactosidase activity (Table 1) showing formation of the conjugate between α-2-M and α-galactosidase. An estimate based on the α-galactosidase activity in α-2-M fraction gave a molar ratio of α-galactosidase versus α-2-M in the mixture as 0.125.

Cultured fibloblasts derived from a patient with Fabry's disease (GM2775 cells) were used to study internalization of the

Table 1. α-galactosidase activity in various preparations of the enzyme

	α-galactosidase activity(μunit)	Amount of protein (μg)	Specific activity (μunit/μg)
Conjugate	240	10	24*
α-2-M:trypsin	none	10	none
α-galactosidase	510	0.09	5670
	44	0.009	4880
	4.3	0.0009	4780

*When one α-galactosidase molecule is conjugated to one α-2-M molecule, the specific activity of the conjugate is to be 190 μunit/μg based on the molecular weights of α-2-M(725,000)(13) and α-galactosidase(26,000)(14). The specific activity of the conjugate given in table indicates that about one in every eight α-2-M molecules carried one molecule of α-galactosidase.

conjugate by determining α-galactosidase activity in the crude cell extracts before and after incubation of the cells with the conjugate. Time dependency of internalization of the conjugate into GM2775 cells is given in Fig.1. When the cells were incubated with Dulbecco's modified Eagle's medium (DMEM) containing 5 mg/ml BSA and 20 μg/ml conjugate (0.547 munit/ml) at 37°C, α-galactosidase activity in the crude cell extracts increased with time. No such increse in enzyme activity was observed when the cells were incubated at 4°C. Furthermore, when the cells were incubated at 37°C with DMEM-BSA containing

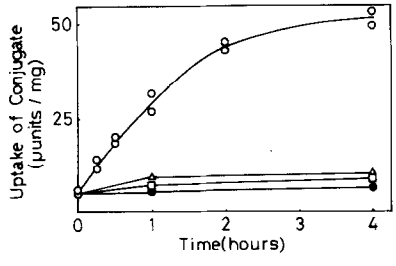

Fig.1. Time-dependency of uptake of the conjugate by GM2775 cells. Each medium contained ingredients given below and incubation was carried out in each medium at the indicated temperature : DMEM-BSA containing 20 μg/ml conjugate at 37°C(-○-) and at 4°C(-●-) ; DMEM-BSA containing 20 μg/ml conjugate and 1 mg/ml bacitracin at 37°C(-△-) ; DMEM-BSA containing 20 μg/ml trypsin-reacted α-2-M and 0.235 μg /ml α-galactosidase at 37°C(-□-).

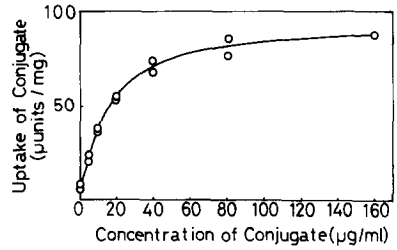

Fig.2. Dose-dependecy of the internalization of the conjugate by GM2775 cells. The cells were incubated at 37°C for 2 h in DMEM-BSA containing the conjugate at various concentrations. Spcific activity of the α-galactosidase was represented as μunits/mg of protein.

20 μg/ml conjugate and 1 mg/ml bacitracin, no significant increase in α-galactosidase activity was observed in the cell extracts. Bacitracin is known to inhibit binding of α-2-M to its receptor but not nonspecific binding of α-2-M to cell surfaces(12). Thus it was shown that the significant increase of α-galactosidase activity in the cell extracts observed in the absence of bacitracin was due to actual internalization of the conjugate via receptor-mediated endocytosis of α-2-M, but not due to nonspecific binding of the conjugate to the cell surface.

When the cells were incubated with DMEM-BSA containing a mixture of trypsin-reacted α-2-M (20μg/ml) and non-conjugated α-galactosidase from coffee beans (0.235 μg/ml) at 37°C, no distinct increase of α-galactosidase activity in the cell extracts was observed, though α-galactosidase activity (2.49 munit/ml) in the mixture was higher than that of the conjugate (0.547 munit/ml). α-galactosidase from coffee beans could not effectively be internalized in unconjugated form.

Dose-dependency of internalization of the conjugate into GM2775 cells is given in Fig.2. The cells were incubated for 2 h in DMEM-BSA containing varying concentrations of the conjugate. With increasing concentrations of the conjugate, the α-galactosidase activity in the cell extracts increased and reached a plateau at around 40 μg/ml of the conjugate. The

observed saturation behavior of α-galactosidase activity in the cell extracts indicated that the conjugate was internalized by the cells via receptor-mediated endocytosis.

The present study showed that the conjugate of α-2-M and α-galactosidase can be prepared by exploiting a unique property of α-2-M. This procedure does not involve modification of proteins with cross-linking reagents which sometimes affect the protein structure and its activity. Our study has clearly shown that the α-galactosidase activity in GM2775 cell extracts increases when the cells are incubated with α-2-M: α-galactosidase conjugate. For the future application of our strategy to enzyme replacement therapy, further studies are needed as to, for example, the antigenicity of the conjugate, effectiveness of replaced enzymes in the degradation of accumulated substrates in the cell. When such studies are completed, our model study for the internalization of deficient enzymes into cultured cells will prove to be a very promising strategy in the enzyme replacement therapy of lysosomal diseases. Moreover, since α-2-M can be conjugated with a variety of proteins and peptides by the same procedure as the one used in the present study, the method we developed here will be a useful way to inject desired proteins and peptides, e.g. growth factors, hormones or oncogene products, into the cells with receptors for α-2-M.

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