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Reaction of 4-Methylumbelliferylguanidinobenzoate with Cultivated Human Skin Fibroblasts Derived from Patients with Cystic Fibrosis*

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Summary

Protease activity in cultivated human skin fibroblasts has been quantitated using 4-methylumbelliferylguanidinobenzoate (MUGB), an active site titrant of trypsin-like proteases (7). The reaction of the proteases with MUGB was complete in 1 hr, inhibited both by benzamidine and (*p*-nitrophenyl)-*p'*-guanidinobenzoate, but not by *p*-hydroxymecuribenzoate. The extent of reaction was proportional to protein concentration and independent of MUGB concentration. This activity was present in the particulate fraction of the cell.

The mean "titre" values (nanomoles of 4-methylumbelliferone released per mg protein) of the proteases in fibroblasts from eight controls (N), 8 obligate heterozygotes (H), and 14 patients with cystic fibrosis (CF) were: N, 1.27 ± 0.11; H, 0.82 ± 0.12; CF, 0.66 ± 0.10. The differences in the "titre" values for N:CF and N:H were significant ($p < 0.001$) as were those for H:CF ($p < 0.01$). The mean "titre" value obtained for cultivated control amniotic fluid cells was 1.29 ± 0.17.

These data indicate a reduction in the MUGB-reactive proteases in skin fibroblasts derived from patients with CF when compared either to control or to obligate heterozygotes. These data are consistent with our earlier suggestion (11, 15) that decreased proteolytic levels in the tissues and fluids of patients with CF may be a generalized phenomenon.

Speculation

The reduced MUGB reactivity of proteases prepared from cultivated skin fibroblasts derived from patients with cystic fibrosis and "titre" values previously reported for plasma proteases from patients with cystic fibrosis suggests that this reduction in protease level is a generalized phenomenon in cystic fibrosis.

INTRODUCTION

We have previously reported that saliva and plasma of patients with cystic fibrosis (CF) is deficient in the enzyme activity hydrolyzing esters of arginine (12,14), and documented the proteolytic nature of the activity (14). Recently, we have reported that this reduction in protease activity can be demonstrated using 4-methylumbelliferylguanidinobenzoate (MUGB), an active site titrant of trypsin-like proteases in chloroform-ellagic acid activated plasma of patients with cystic fibrosis (11,15). There was a concomitant decrease in proteolytic activity towards esters of arginine in these activated plasma samples, as has previously been described (14). It was also reported that unactivated plasma samples reacted with MUGB to the same extent (nmoles 4-methylumbelliferone released per ml plasma) as did the activated samples with significant differences in the titre values maintained between control, obligate heterozygotes, and patients with CF. These unactivated plasma samples had negligible catalytic activity towards the classical ester substrates derived from L-arginine.

Until recently, it had not been possible to demonstrate a similar reduction in protease activity in cultivated skin fibroblasts derived from patients with cystic fibrosis using conventional substrates, probably due to the insensitivity of the procedures. We now present data on the determination of protease activity in cultivated human skin fibroblasts using MUGB and the demonstration of a reduction in this activity in fibroblasts derived from patients with cystic fibrosis. Furthermore, we also present data on the quantitation of this activity in cultivated human amniotic fluid cells.

MATERIALS AND METHODS

Chemicals

Benzamidine·HCl and 4-methylumbelliferylguanidinobenzoate (MUGB) were purchased from Sigma Chemical Company (16). Dimethylsulfoxide used as the solvent for MUGB was obtained from J.T. Baker Chemical Company (17). (p-nitrophenyl)-p'-guanidinobenzoate (NPGB) was purchased from Nutritional Biochemical Corporation (18). p-hydroxymecuribenzoate (HMB) was from Schwartz/Mann (19).

Source of Human Skin Fibroblasts and Amniotic Fluid Cells

Human skin fibroblasts were obtained either from stock cultures in our laboratory or from the Human Genetic Mutant Repository (20). Skin biopsies from patients with CF, obligate heterozygotes, and control subjects were obtained with informed consent under the guidelines of the Department of Health, Education, and Welfare and with the approval of the Human Ethics and Research Committee of the Children's Memorial Hospital. Human amniotic fluid cells obtained at approximately sixteen weeks of pregnancy were from routine amniocentesis.

Cell Cultures

Human fibroblasts were grown from skin biopsies and amniotic fluid cells were cultivated by standard methods as previously described (10). Experimental use of skin fibroblast lines was discontinued after 20 passages. For the growth experiments, minimal essential medium containing 15% fetal calf serum with penicillin, streptomycin, and fungizone added was changed on the third and seventh day after initial planting. The fibroblasts were harvested in saline by scraping and the homogenates prepared as described below.

Preparation of Cell Homogenates

Human skin fibroblasts and amniotic fluid cells were harvested by scraping the flasks with a rubber policeman. Cells being prepared for assay with MUGB were not harvested with trypsin, which has been shown to react with MUGB (7). The cells were washed with 0.85% saline and spun in a clinical centrifuge for 15 minutes at 4°C. This procedure was repeated three times. The washed cells were resuspended in 0.01 M Veronal HCl-saline solution, pH 8.3. Usually, cells from six flasks were resuspended in 0.4-0.6 ml of the veronal-saline solution for sonication. The cells were homogenized for one minute. The microtip of the sonifier (Heat Systems Ultrasonics, Inc., Model W 185 (21)) was set to maximum and the suspension sonicated in ice in bursts of ten seconds. The homogenate was spun at 600 x g in a clinical centrifuge for 15 minutes at 4°C and the low speed supernatant used in the assay system. The low speed supernatant was used either immediately or stored at -10°C. Storage for up to thirty days did not affect the assay. The washed pellet obtained from the centrifugation was found to react with MUGB; however, most of the 4-methylumbelliferone (4-MU) released (85-90%) was due to non-specific hydrolysis of the substrate.

Assay Methods

The reaction of low speed supernatants obtained from cultivated skin fibroblast and amniotic fluid cell homogenates was performed as previously described for plasma (11,15) with a few modifications. 0.20 µl of the supernatant (1-3 mg protein per ml) was incubated for varying time periods with 0.20 µl of either distilled water or 0.2 M benzamidine·HCl and 0.20 µl of either 0.10 µM or 0.20 µM solution of MUGB with 4% dimethylsulfoxide in 0.10 M veronal, pH 8.3 at room temperature in the dark. The reaction was terminated by the addition of 2 ml of the veronal buffer and the 4-MU liberated was determined fluorometrically (Aminco Bowman Spectrofluorimeter (22)) with excitation at 365 nm and emission at 450 nm. Appropriate blanks were run in parallel to correct for the spontaneous liberation of 4-MU. Each time point was routinely done in triplicate. Protease activity was taken as the difference between the amount of 4-MU liberated in the presence and absence of 0.2 M benzamidine, documented to inhibit plasma proteases known to be deficient in cystic fibrosis (11,15). The 4-MU reaction values obtained in the presence of benzamidine were presumably due to the non-specific hydrolysis of the substrate. The 4-MU liberated in the presence and absence of benzamidine is also corrected for the spontaneous hydrolysis of MUGB by substituting buffer for the supernatant in the reaction mixture. Since the amino acid side chains present in tissue proteins hydrolyze MUGB nonspecifically to a greater extent than when they are coagulated by boiling, use of benzamidine in presence of soluble protein is a better approach than using boiled blanks to determine the contribution due to proteolytic activity.

The extent of reaction of MUGB with proteases in the supernatants obtained from human skin fibroblasts and amniotic fluid cells is expressed as nmoles 4-MU released per mg protein. Protein determinations were made with bovine serum albumin as standard according to the method of Lowry et al. (9).

RESULTS

Standardization of Assay Conditions

Fibroblast low speed supernatants contain activity hydrolyzing MUGB. This reaction is complete in one hour. The activity is inhibited by benzamidine (Fig. 1) and NPGB and present in the particulate fraction of the cell. This protease activity is destroyed by boiling.

The extent of reaction is proportional to protein concentration over a two-hour incubation period and independent of MUGB concentration (0.025 µM-0.20 µM). When MUGB reactivity in the supernatants of one control and one cystic fibrosis fibroblast lines are compared, titration curves similar to the one in Figure 1 are obtained. A significant difference in the amount of 4-MU liberated is seen when the "titre" values of the supernatants from the control and cystic fibrosis cell lines are compared. However, the amount of 4-MU liberated during the course of the non-specific reaction in the presence of benzamidine is approximately identical for both samples (Fig. 1).

Effect of Culture Conditions on MUGB Reactivity

The growth and protease activity patterns of one control and one cystic fibrosis fibroblast line are shown in Figure 2. Protein content per flask became constant on the 8th day and media change on the 7th day had no significant effect at this stage (Figure 2a). Protein per flask was approximately the same for both lines. However, the protease activity expressed either as total activity per flask (Fig. 2b) or as specific activity (Fig. 2c (nmole 4-MU liberated per mg protein)) was lower in the fibroblast line derived from a patient with cystic fibrosis at all stages of growth. This difference became significantly increased after the sixth day. Therefore, comparisons in the protease activity between control and cystic fibrosis cell lines have been made using fibroblasts harvested at the seventh or eighth day.

Reaction of Supernatants of Fibroblast Homogenates from Control, Obligate Heterozygotes and Cystic Fibrosis and Control Amniotic Fluid Cells with MUGB

A comparison of protease activity in the low speed supernatants obtained from control, obligate heterozygotes, and cystic fibrosis cell lines is shown in Figure 3. Significant differences were observed in the extent of reaction of the supernatant proteases with MUGB between the controls (N), obligate heterozygotes (H) and cystic fibrosis (CF) samples. The mean "titre" values (nmoles 4-MU released per mg protein) which are corrected for non-specific hydrolysis using benzamidine from 8 N was 1.27 ± 0.11; 8H, 0.82 ± 0.12 and 14 patients with CF, 0.66 ± 0.10. The "titre" values for N:CF and N:H were significantly different with a p value of less than 0.001 and for CF:H, 0.01. The mean total 4-MU liberated by the supernatant proteases of CF lines is approximately 50% that of controls. Titration experiments using low speed supernatants obtained from cultivated human amniotic fluid cell homogenates have shown that the reaction is inhibited by benzamidine and the mean "titre" value is 1.29 ± 0.17 nmoles 4-MU released per mg protein (Fig. 3).

Mixing equal volumes of fibroblast supernatant samples from controls and patients yielded the expected intermediate values for the 4-MU liberated.

DISCUSSION

These data indicate a significant reduction in the number of protease molecules which react with the titrant, MUGB, when the low speed supernatants from cultivated skin fibroblast homogenates dervied from patients with cystic fibrosis are compared to controls (Fig. 3). Mixing experiments ruled out the presence of any inhibitor in samples from fibroblasts dervied from patients with cystic fibrosis. This is not surprising, since even strong inhibitors like soybean trypsin inhibitor had no effect on the reaction of MUGB with plasma proteases, due to the high affinity of MUGB to the active sites of the enzymes. Previous work from this laboratory demonstrated a deficiency of protease activity in plasma and saliva of patients with cystic fibrosis by measuring the rate of hydrolysis of esters of L-arginine (13,14) and protamine (14). Recently, we demonstrated a reduction in the amount of MUGB-reactive proteases in both chloroform-ellagic acid activated and unactivated plasma from patients with cystic fibrosis (11,15). MUGB has been employed as an active site titrant in both pure (7,8) and crude (2) systems. It also was found to be a very sensitive indicator of STI-inhibited protease molecules in plasma (11,15). In plasma, it was suggested that the differences in the mean "titre" values (nmoles 4-MU released per ml plasma) between cystic fibrosis and control plasmas represented a reduced number of protease molecules in cystic fibrosis rather than a decrease in their catalytic efficiency. Similar observations are apparent in the MUGB "titration" assays with cultivated human skin fibroblast proteases.

Reaction characteristics similar to those reported for plasma (11,15) are observed when the low speed supernatants prepared from cultivated human skin fibroblast homogenates were reacted with MUGB. The reaction is essentially complete in 60 minutes (Fig. 1) similar to that reported for both plasma (11, 15) and thrombin (7) but much slower than that reported for trypsin and Factor X_a (7). It is proportional to protein concentration, independent of MUGB concentration and inhibited by both NPGB, an active site titrant of trypsin and the nitrophenyl analog of MUGB (4,5) and benzamidine, a highly specific, competitive inhibitor of trypsin-like proteases (5). Though non-specific hydrolysis is indicated by the formation of 4-MU in the presence of both benzamidine (Fig. 1) and NPGB, the amount of non-specific hydrolysis was found to be the same for both the control and cystic fibrosis cell line (Fig. 1). The reaction of MUGB with fibroblast supernatants probably involves a mixture of proteases. The release of 4-MU may be due to "titration" of some of these proteases and the turnover of MUGB as a substrate by others. Although the conbribution of each of these components cannot be clearly identified at present, the strong affinity of MUGB for the active site of many proteases (2,7,8) and the sensitivity of fluorometry makes this approach extremely adaptable to small amounts of trypsin-like proteases known to be important in cystic fibrosis (11-15). It is also possible that the fibroblast proteases are in an inactive, precursor form, in which case synthetic substrates would not be effective; but MUGB, which is known to react with trypsinogen (8) and protease precursors in plasma (11,15) might react with both precursors and active forms of proteases in fibroblasts.

The nature of the proteolytic activity which is present in cultivated human skin fibroblasts and "titrated" with MUGB is not known. However, it is not the plasminogen activator, a serine protease (6), because the MUGB-reactive species is neither soluble nor is it in our experiments easily solubilized. HMB, an inhibitor of thiol proteases (1) has no effect on the liberation of 4-MU in this assay system similar to results reported for plasma (14),

indicating it is not thiol protease such as cathepsin B (1). A soluble protease with arginine esterase activity has recently been described in liver (3). The protease was unaffected in patients with cystic fibrosis. However, it was not inhibited by soybean trypsin inhibitor and had a pH optimum different from those proteases known to be deficient in patients with CF (11-15). The reaction of fibroblast proteases is inhibited both by benzamidine (Fig. 1) and NPGB indicating the involvement of trypsin-like molecules and it is thought that the MUGB-reactive enzymes are probably neutral proteases associated with the plasma membranes. The exact nature of this activity remains to be defined.

The techniques of quantitation of fibroblast proteases with MUGB has been demonstrated to be both very sensitive and reproducible, and can detect very low levels of proteases hitherto very difficult to demonstrate using standard rate assays. The reduced levels of proteases in saliva (12), plasma (11, 13-15), and cultivated human skin cell fibroblasts derived from patients with cystic fibrosis suggests that this reduction in activity is a generalized phenomenon in cystic fibrosis. Refinement of these techniques may permit heterozygote and intrauterine detection.

CONCLUSION

A reduction in cellular proteases assayed using MUGB has been demonstrated in the low speed supernatants prepared from cultivated skin fibroblast homogenates derived from patients with CF. Significant differences are observed in the extent of reaction of fibroblast proteases with MUGB when comparisons are made between control and cystic fibrosis cell lines. The mean "titre" value (nmoles 4-MU released per mg protein) obtained for the fibroblast lines derived from patients with CF is approximately 50% of that of the normal lines. MUGB reactivity has also been documented in cultivated control amniotic fluid cells. These data are consistent with our earlier suggestion that decreased proteolytic activity in the plasma of patients with cystic fibrosis may be the result of fewer protease molecules rather than differences in their catalytic efficiency (11,15). This apparent reduction in protease levels would seem to be a generalized phenomenon in the tissues and fluids of patients with cystic fibrosis.

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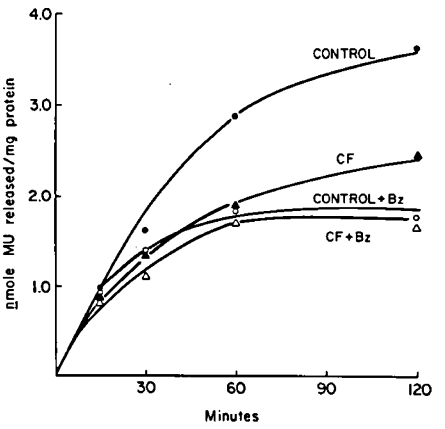

Fig. 1. Reaction of supernatants of fibroblast homogenates from a control and a CF cell line with MUGB.

Time course of the reaction of MUGB and proteases present in the supernatants of fibroblast homogenates derived from a control (●) and a patient with CF (▲). Parallel samples were run in the presence of 0.2M benzamidine. Control + benzamidine (o), CF + benzamidine (Δ). The reaction is not a rate assay and levels off after 60 minutes. The nature of the reaction meets many of the criteria for an "active site titration" (see Text and Refs. 2,4,5,7,15).

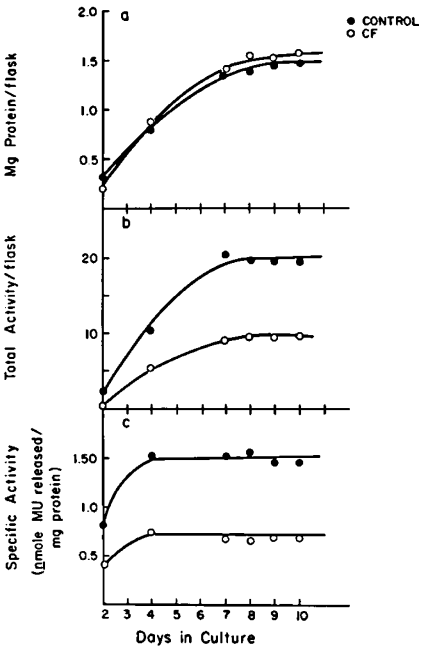

Fig. 2. Protease patterns of a control and a CF fibroblast line as a function of growth conditions.

Growth (a) and the reaction of fibroblast proteases with MUGB (b,c) from a control (●) and a CF (o) cell line assayed at various periods after initial planting of cells.

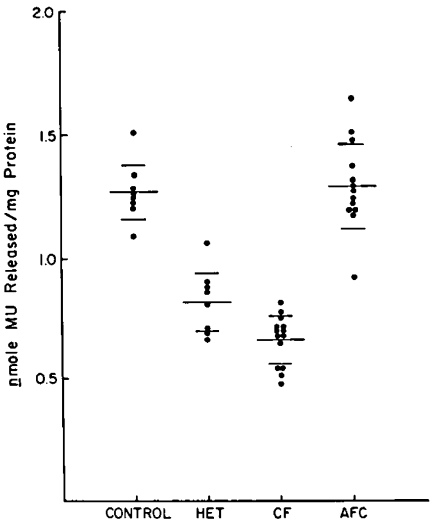

Fig. 3. Scattergram of the "titre" values obtained when supernatant proteases from control, obligate heterozygote and CF fibroblast and control amniotic fluid cell homogenates are reacted with MUGB.

Obligate heterozygote, Het; amniotic fluid cells, AMF. The large bars indicate the mean and the small bars indicate one SD.

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