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Biodegradable microspheres for prolidase delivery to human cultured fibroblasts

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Abstract

Prolidase deficiency (PD) is a rare autosomal recessive disorder caused by inadequate levels of the cytosolic exopeptidase prolidase (E.C. 3.4.13.9), for which there is not, as yet, a resolutive cure. We have investigated whether biodegradable microspheres loaded with prolidase could release active enzyme inside cells, to consider this system as a possible therapeutic approach for prolidase deficiency. Poly(lactide-co-glycolide) microspheres were prepared, modifying the classical double emulsion solvent evaporation method to mitigate the burst effect of the enzyme from the microspheres. Ex-vivo experiments were performed, by incubating microencapsulated prolidase with cultured fibroblasts from PD patients and from controls, to determine the amount of active enzyme delivered to the cells. The microparticulate drug delivery system described carried small amounts of active prolidase inside fibroblasts, ensuring a response to the intracellular accumulation of X-Pro dipeptides, the mechanism that is supposed to be responsible for the development of clinical manifestations of this disorder in man. A positive result of the presence of active enzyme inside cells was an improvement in fibroblast shape.

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Introduction

Prolidase (E. C. 3.4.13.9) is a cytosolic exopeptidase that cleaves imidodipeptides and imidotripeptides with C-terminal proline or hydroxyproline (Yaron & Naider 1993). A rare autosomal recessive disorder causing deficiency of this enzyme in man is characterized by massive urinary excretion of imidodipeptides, X-Pro and X-Hyp (Royce & Steinmann 2002). Although there are descriptions of some subjects with prolidase deficiency (PD) who do not have the clinical features (Isemura et al 1979; Milligan et al 1989), common manifestations of this disorder are severe skin ulceration of the lower limbs, mental retardation and frequent infections of the respiratory tract. The complexity of such manifestations makes these patients' daily life rather difficult and, although a variety of therapies have been attempted (Arata et al 1979, 1986; Charpentier et al 1981; Pedersen et al 1983; Yasuda et al 1999), a resolutive cure for this disease has not yet been found. Although transient beneficial effects were recently observed in a PD patient submitted to replacement therapy with reduced glutathione (GH) and topical application of this compound on skin ulcerations (Monafo et al 2000), it is not yet clear whether enzyme replacement therapy based on blood transfusions can play a major role in the treatment of PD. In fact, in one case in which this strategy was used (Endo et al 1982), the urinary level of imidodipeptides remained unchanged although the prolidase activity of erythrocytes did rise to 35% of normal values. In contrast, a patient who had been submitted to blood transfusions in our department (Berardesca et al 1992) had ulcers on the feet that diminished in size, although the increase of enzyme activity was very low. In an attempt to rationalize this form of replacement therapy we performed repeated apheresis erythroexchanges using continuous flow equipment in two patients, in parallel, for four consecutive months (Lupi et al 2002). Although the constant presence of active prolidase inside cells allowed continuous breakdown of imidodipeptides, with an apparent concomitant improvement of skin ulceration, the question of whether partial healing of the ulceration justified the use of a chronic, hazardous and expensive therapy is an important

issue that still needs to be answered. To overcome these problems we have evaluated the possibility of replacing the deficient enzyme through parenteral administration of prolidase encapsulated in biodegradable microspheres. Microparticulate drug delivery systems based on poly (D,L-lactide-co-glycolide) have been extensively studied recently and they have been demonstrated to be efficient carriers for polypeptides and proteins (Jiang et al 2003; Sinha & Trehan 2003). In a previous report (Genta et al 2001) we defined the experimental conditions needed to preserve prolidase activity during its encapsulation into microspheres of poly (D,L-lactide-co-glycolide) (PLGA). The procedure provided stable enzyme activity in-vitro and in the presence of cellular extracts from human fibroblasts.

The aim of this study was to investigate whether prolidase released from microspheres entered the cells and to determine the efficiency of this transcellular delivery, to consider this system as a possible therapeutic approach for prolidase deficiency.

PLGA-microspheres were prepared using a modification of the previously reported double emulsion solvent evaporation method (Genta et al 2001) to achieve a lower burst effect of the enzyme from the microspheres.

The morphology, drug loading and dissolution of these protein-loaded microspheres were characterized in-vitro. Ex-vivo experiments were performed in which microencapsulated prolidase was incubated with cultured fibroblasts from patients affected by prolidase deficiency and from healthy volunteers as controls to determine the amount of active enzyme delivered to cells.

Materials and Methods

Materials

Poly (D,L-lactide-co-glycolide) Resomer RG 503H, inherent viscosity 0.38 dL g^{-1} M_R 34 000 Da, polydispersity index 3, range 2.63 to 3.21 was obtained from Boehringer (Ingelheim am Rhein, Germany).

Prolidase from pig kidney (freeze-dried powder containing 30.9 U mg^{-1} of active protein) was obtained from Biozyme Laboratories (Blaevon, UK). Bovine serum albumin (BSA), poly (vinyl alcohol) (PVA) 87–89% hydrolysed, 85 000–146 000 Da, glutathione reduced form (GSH), tris[hydroxymethyl]aminomethane (Tris), gly-pro substrate and polyethylene glycol (PEG), 400 Da, manganous chloride, dodecyl sulfate sodium salt (SDS), Dulbecco's modified Eagle's medium (DMEM) and insulin-transferring sodium selenite (ITS+3) serum substitute used for fibroblasts cultures were purchased from Sigma (Milan, Italy). Bicinchoninic acid (BCA) Protein Assay Reagent Kit was obtained from Pierce (Rockford, IL) and fetal calf serum (FCS, Euroclone) was obtained from Celbio (Milan, Italy).

The composition of the working solutions used for the experimental procedures were as follows: solution A, 50 mM Tris HCl, pH 7.8 containing 100 mM $MnCl_2$, and 1 mM freshly prepared GSH; solution B, 80 mM gly-pro peptide in 50 mM Tris HCl pH 7.8; solution C, 0.45 M trichloroacetic acid (TCA).

Microsphere preparation

The enzyme-loaded microspheres, theoretically containing 10 mg of commercial prolidase, were prepared utilizing the w/o/w double emulsion solvent evaporation method set up in a previous work (Genta et al 2001). Solid microspheres were recovered by centrifugation followed by filtration through a 0.45-μm Millipore membrane.

Although the double emulsion solvent evaporation method is commonly used to load proteins into microspheres (Chen et al 2002), it frequently results in the production of microspheres with a high burst effect because most of the protein resides at the surface of these particles. In this work four different compositions of the external aqueous phase were used. Batch 1, PVA 1% w/w, deionized water; batch 2, PVA 1% w/w, NaCl 10%, deionized water; batch 3, PVA 1% w/w, NaCl 2%, deionized water; and batch 4, PVA 1% w/w, citrate buffer pH 4.2. The microspheres obtained were compared in terms of their dissolution behaviour.

When NaCl was added to the external aqueous phase (batches 2, 3), PEG 400 was added to the internal phase containing the protein, with the aim of protecting the protein from denaturation caused by NaCl, as reported by Schwendeman (2002). Addition of citrate buffer to the external outer phase (batch 4) produced a pH close to the isoelectric point (pH 4.5) of prolidase, thus creating an environment unfavourable to the exit of protein from the internal phase during preparation of the microspheres.

Batch 1 was prepared without any modification to the standard procedure (Genta et al 2001), and was used as the control.

Microsphere characterization

In-vitro characterization of the microspheres involved morphologic analysis, determination of total protein content, dissolution tests and protein activity evaluation. The morphology of the microspheres was examined by scanning electron microscopy (SEM) with a Cambridge Stereoscan 200 electron microscope (Cambridge Ltd, Cambridge, UK). Samples were prepared by placing suitable amounts of microspheres on the microscope sample holder and then sputtering them with gold in an Argon atmosphere.

Granulometric analysis was performed using a Coulter Multisizer II apparatus (Coulter Electronics Ltd, Luton, UK). Samples of microspheres were suspended in 0.9% NaCl filtered aqueous solutions and analysed under continuous stirring.

Tests were performed for determination of the total protein loaded and/or released from microspheres (BCA assay), and to assess prolidase activity (HPLC assay).

Total protein content was determined after microsphere digestion through 5% SDS solution in 0.1 M NaOH. Following digestion of the microspheres, the clear solutions that contained the protein were purified from salts and polymer by separation on a PD-10 column (Amersham Pharmacia Biotech) with 9.1 mL total bed volume using double distilled water as the eluent. The protein content was determined using the BCA reagent kit (calibration curve drawn between 5 and 250 μg mL^{-1}).

Prolidase activity was determined essentially as previously reported using an HPLC (Hewlett Packard, series 1100) equipped with a C18 column (250 × 4.6 mm i.d., 5-μm pore size) (Genta et al 2001). The mobile phase was 10 mM KH_2PO_4 containing 0.5 mM hexansulfonic acid sodium salt, pH 2.7; the flow rate was 1.9 mL min^{-1}.

In-vitro prolidase release tests were performed on prolidase-loaded microspheres in Eppendorf test tubes by suspending 30 mg microspheres, theoretically loaded with 0.5 mg prolidase, in 500 μL solution A. The test tubes were shaken and maintained at 37 °C in a water bath during the whole dissolution time (40 days). At fixed intervals the suspensions were centrifuged, and 100-μL samples of the supernatants were withdrawn and analysed to determine both the total protein and the active prolidase released.

A stability test on free prolidase (6 IU mL^{-1}) dissolved in solution A was performed as control.

One unit of prolidase activity (IU) was defined as the amount of enzyme that hydrolysed 1 μmol gly-pro in 1 h at 37 °C. Prolidase specific activity (%) was defined as the ratio of active to total enzyme.

Ex-vivo evaluation of prolidase activity on cultured skin fibroblasts

The Institutional Ethics Committee approved this study. Skin biopsies from controls (n = 2) and PD patients (n = 2) were obtained after informed signed consent. Fibroblasts (5×10^5) were plated in T25 flasks containing DMEM supplemented with 10% FCS. After 24 h the initial medium was changed with DMEM containing 1% ITS+3 serum substitute and supplemented with 29 mg microspheres encapsulating prolidase from pig kidney (10 mg corresponding to approximately 30 IU). After three days of incubation, the medium was removed and a 2.5-mL sample desalted on a PD-10 column. The eluting material was lyophilized, taken up in 0.2 mL 50 mM Tris HCl pH 7.8 and divided into two 0.1-mL samples, one of which was used for determination of prolidase activity and the other for quantification of total proteins. The cellular layer was washed twice with phosphate-buffered saline to completely remove possible traces of culture medium. Fibroblasts were trypsinized for 5 min at 37 °C and centrifuged at 1500 *g* for 6 min; the pellet was washed with 50 mM Tris HCl pH 7.8 and the cells resuspended in 0.5 mL of the same buffer. The cellular extract was obtained by submitting this material to a thawing procedure and centrifuging at 12 000 *g* for 20 min. Finally, the supernatant was divided into two samples to determine prolidase activity and total protein.

The values of prolidase activity in medium and in cellular extracts from controls and patients were expressed as μmol proline released h^{-1} (mg protein)$^{-1}$.

Statistical analysis

Data were expressed as mean ± s.d. Comparison of mean values was performed using the *t*-test or the Kruskal–Wallis analysis of variance. Differences were considered statistically significant at $P < 0.05$.

Light microscopy analyses

The morphology of cells from patients and controls was examined with contrast-phase light microscopy using a Lietz Diavert (Jena, Germany) instrument. Images were obtained using a magnification × 20.

Results and Discussion

We had shown previously (Genta et al 2001) that prolidase encapsulated in microspheres was more stable than free enzyme, and had established that enzyme activity was not degraded by endogenous components of fibroblasts.

In this study we aimed to demonstrate that this system could represent a rough model for the future development of an enzyme replacement therapy, because it was effective in delivering, at least partially, prolidase intracellularly. In keeping with this purpose we performed experiments where fibroblasts from controls and patients were incubated with microencapsulated prolidase over different time spans, and assaying prolidase activity on cellular extracts.

Characterization of microspheres

Given that the initial burst effect observed with the microspheres described above was rather high, we attempted to produce a series of microparticles with characteristics slightly different from each other, to obtain a material with better kinetics of enzyme release. The data reported in Table 1 summarize the yields of production and the encapsulation efficiencies (in terms of total protein content) determined on the different types of microspheres prepared.

The data from the granulometric analysis expressed as d50% showed that, on average, microspheres of batches 1 and 4 were larger compared with batches 2 and 3.

Although the dissolution tests were carried out over a 40-day period (data not shown), an interval in which all batches released 100% protein, our attention was focused on the dissolution behaviour of the first six days. This

Table 1 Results of microsphere characterization in terms of yields of production, encapsulation efficiency and granulometric analysis.

Batch no.	Yield of production (% ± s.d.)	Encapsulation efficiency (% ± s.d.)*	d50%** (μm) ± s.d.
1	78 ± 1	36.34 ± 0.5	30.25 ± 0.02
2	74 ± 0.6	34.00 ± 0.8	15.25 ± 0.06
3	80 ± 0.8	35.91 ± 0.6	21.25 ± 0.08
4	74 ± 0.8	25.15 ± 0.8	32.12 ± 0.04

*Encapsulation efficiency was calculated from the ratio between the total amount of protein content, as determined by BCA assay, and theoretical amount of proteins (prolidase and BSA) loaded in microspheres. **d50% value represents the size of 50% of particles as determined by Coulter Counter analysis.

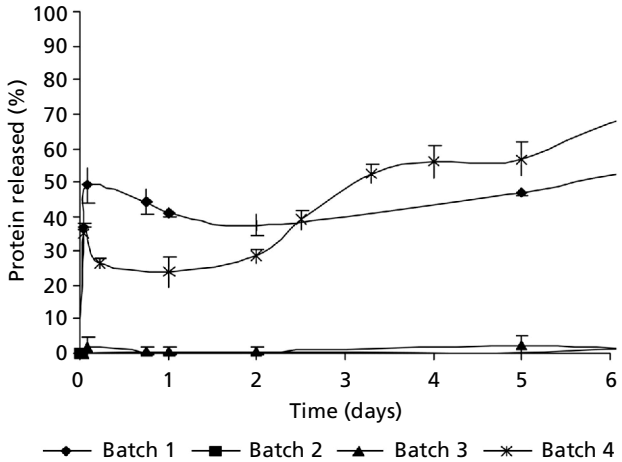

Figure 1 In-vitro protein release profiles from PLGA microspheres (batches 1–4) over six days.

interval was suitable for performing in-vivo experiments, to correlate the data relative to total protein (prolidase plus BSA) release with those of enzyme activity.

As shown in Figure 1, the amount of protein released from batches 2 and 3 during the first six days was very low and not significantly different from batch to batch ($P > 0.05$). Given that we were searching for biomaterials capable of releasing the encapsulated protein in a short period, we considered these batches unsuitable for our purposes. By contrast batch 1 showed an initial high burst effect followed by a plateau. This burst effect, although present in batch 4, was moderately reduced in this latter batch by the addition of citrate buffer to the external aqueous phase, probably because of an improvement of protein encapsulation inside the polymeric matrix. Statistical analysis (a *t*-test was applied) of the dissolution data relative to the microspheres of batches 1 and 4 demonstrated that if the total amount of protein released from batch 4 was significantly lower ($P < 0.05$) compared with batch 1 within the first two days of the process, it was significantly higher ($P < 0.05$) on days 4 to 6. This suggested that batch 4 could represent the best system for delivering prolidase to the cell.

Release of active prolidase from microspheres of batches 1 and 4

Evaluation of active prolidase released from microspheres, over a 6-day dissolution period, confirmed that active enzyme was released from batch 1 immediately (data not shown), while a gradual profile of active prolidase was obtained for batch 4. The release rate of active enzyme reached a maximum after approximately two days (Figure 2A) with low burst effect. This suggested that most of the enzyme was encapsulated inside the microsphere matrix. The morphologic characteristics of these microspheres (Figure 2B) could contribute to the prolonged release of the enzyme since they were bigger than those of other batches and had a compact matrix with low surface porosity.

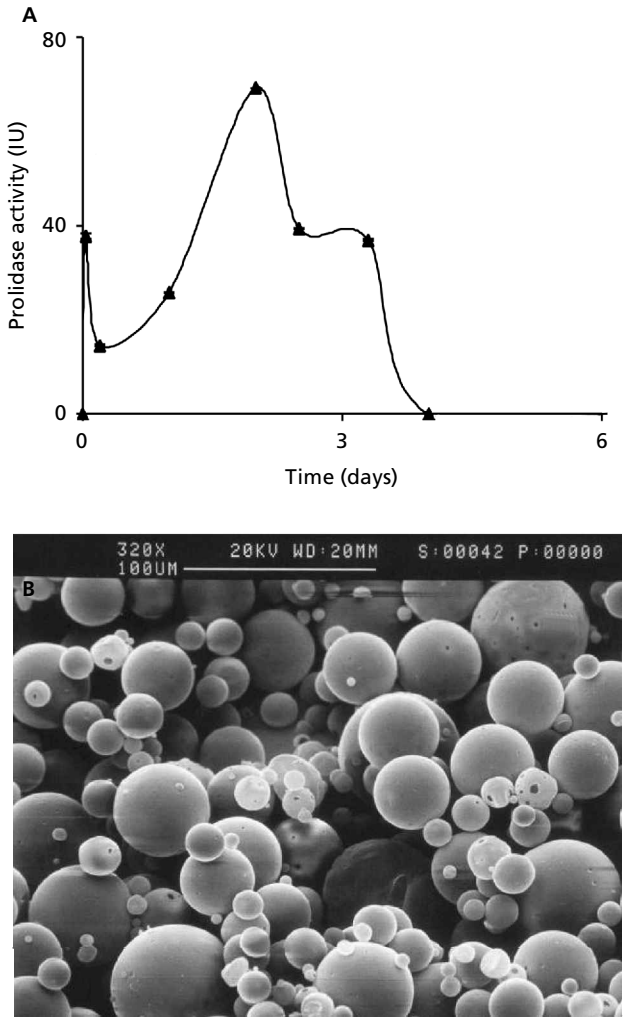

Figure 2 A. Activity profile of prolidase released in-vitro from PLGA microspheres (batch 4). Results are mean ± s.d. from three independent determinations. Absence of error bars indicated that the s.d. fell within the symbol dimensions. B. Photomicrograph of prolidase-loaded PLGA microspheres (batch 4).

Incubation of microencapsulated prolidase with skin fibroblast cultures

Given the good performance offered by microspheres of batch 4, we used these to carry out a series of experiments in ex-vivo systems to investigate whether prolidase released from these microspheres could enter the cells. Fibroblasts from normal subjects and PD patients were incubated in the absence or in the presence of microspheres, which either encapsulated or lacked prolidase. Enzyme activity determined in cellular extracts was compared with that found in culture media following the procedure previously indicated. To account for any disparities in cell numbers in cultures from the controls and the patients, the data on prolidase activity were presented as IU (mg total protein)$^{-1}$ and were the means ± s.d. of four experiments, each performed in triplicate. As far as culture media of controls were concerned, data were collected "in parallel", after a

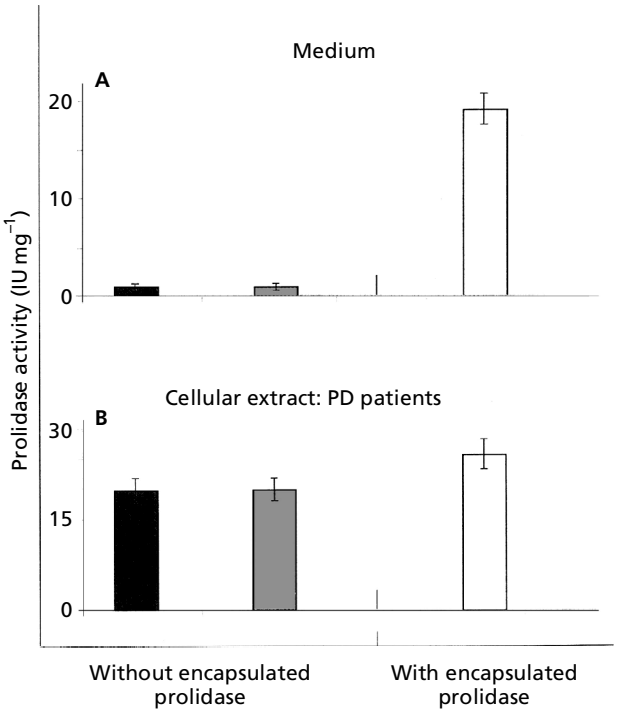

Figure 3 A. Prolidase activity determined in culture media of skin fibroblasts alone or in the presence of microspheres lacking prolidase (left, bars in black and grey, respectively) and in media of the same fibroblasts but incubated with encapsulated prolidase (right). B. Prolidase activity determined on cellular extracts from fibroblasts of two PD patients, cultured in the absence or in the presence of microspheres lacking prolidase (left, bars in black and grey, respectively) and in the presence of microencapsulated prolidase (right). Results are means ± s.d. from four experiments, each performed in triplicate.

3-day incubation period, from cultures containing fibroblasts alone or in the presence of microspheres lacking prolidase (both used as blanks) and from cultures in which fibroblasts were incubated with microencapsulated enzyme. Prolidase activity (19.12 ± 1.5 IU mg^{-1}; Figure 3A, right-hand side) determined in the culture medium from controls treated with microencapsulated enzyme, was approximately 20-fold higher than that obtained in the two blanks in which, as expected, it was practically absent (0.49 ± 0.1 IU mg^{-1}; Figure 3A, left-hand side). The analysis of data performed using the Kruskal–Wallis test produced a KW = 11.47 with a $P < 0.01$.

The amount of prolidase released in this culture medium, calculated taking into account the loss of prolidase activity incurred during the various steps of the microencapsulation procedure, accounted for approximately 63% of the theoretical units initially added to the microspheres. Similar results (not shown) were obtained by analysing culture media from patients: prolidase activity measured in the fraction to which encapsulated enzyme was added was found to be 21-times higher than that of blanks ($P < 0.01$), and the total amount of enzyme released from microspheres was calculated to reach 58% of the theoretical units incubated. These results established that the behaviour of microspheres in this system was in good concordance with that observed in-vitro. However, since the main purpose of this study was the development of a model for carrying prolidase into the fibroblasts, our attention was focused particularly on understanding whether and how our system could deliver prolidase to the cells. Thus, fibroblasts isolated from the above mentioned culture media were processed as indicated in the experimental section and the prolidase content of the different cellular extracts was analysed separately.

The results of experiments performed on PD patients (Figure 3B) showed that the amount of prolidase detected in cellular extracts of fibroblasts incubated with microencapsulated enzyme was higher (right-hand side) than that of cells incubated without encapsulated prolidase, the intracellular enzyme activity being increased by approximately 49% (from 18.02 ± 1.5 to 26.84 ± 1.9 IU mg^{-1}, $P < 0.01$) in fibroblasts of these patients, in which its basal level was very low (Figure 3B, left). A very small increase (from 206.5 ± 15 to 220.8 ± 20.1 IU mg^{-1}, $P > 0.05$), ranging within the experimental error of the method, was observed in fibroblasts of controls. To understand whether the level of prolidase activity carried into the fibroblasts could be directly correlated to the amount of exogenous microencapsulated enzyme added to the incubation mixture, we performed experiments in which fibroblasts from patients were incubated for three days with scalar amounts (5, 10, 20 and 30 IU) of microencapsulated prolidase. The intracellular level increased proportionally with the amount of encapsulated enzyme added (in the range from 5 to 20 IU) until a threshold of 26.90 ± 1.8 IU mg^{-1} was reached. The level then remained practically constant despite the fact that the amount of exogenous enzyme added via the microspheres was increased further to 30 IU. It is unclear whether this saturated uptake depended on the existence of factors regulating the balance of prolidase concentration across the cell membrane, or whether the uptake of microparticles might have reached the saturation uptake and hence there was no increase in the enzyme activity with the increase in the microparticle dose. On the basis of data collected, the amount of exogenous prolidase internalized in fibroblasts of patients was calculated to be approximately 30% of that theoretically encapsulated. This value, taken together with that determined in culture media, confirmed that the vehicle used to deliver the prolidase protected the activity of the enzyme well, resulting in a recovery of prolidase ranging from 70 to 80% of the theoretical units microencapsulated.

Although confirming that exogenous prolidase could indeed be transferred across the cell membrane, these data did not elucidate the mechanism by which this event occurred. In fact, most reports published so far (Panyam & Labhasetwar 2003; Rejman et al 2004) have demonstrated that only particles $< 1\,\mu$m in size are internalized. Thus, given that a pathway of entry for particles $> 1\,\mu$m in size has never been shown, an alternative hypothesis is that the loaded drug should be released outside the cell and cross the membrane by means of a specific receptor. However, although the existence of a prolidase-recognition molecule

that might function as a transmembrane receptor could be proposed as a possible mechanism to explain this transport, evidence for the presence of such a cell-specific system for prolidase has never been provided. Since this question is still a matter of speculation, our results could provide a larger context for future studies devoted to corroborating this view.

Light microscopy analyses

To verify whether this internalization of exogenous prolidase, although partial, could produce beneficial effects on cells from PD patients, we performed light microscopy analyses of cultured skin fibroblasts. The photograph of cells from PD patients taken at time zero of the experimental procedure i.e. immediately after the addition of 20 IU microencapsulated prolidase (black spots in the figure) is illustrated in Figure 4A. As shown, the morphology of these cells, round and branched with disorganized orientation, is clearly different from that of the controls (Figure 4B) that appear more polygonal. After 3-day incubation with prolidase-loaded microspheres, most likely in response to the internalization of exogenous prolidase released following disgregation of particles, the sick cells developed the typical tapered shape and parallel orientation of fibroblasts shown in Figure 4C, appearing similar to, and even better than control cells. These morphological changes were not observed in cells from PD patients not treated with microencapsulated prolidase (not shown) nor in those treated for the same period with microspheres lacking prolidase (Figure 4D). Therefore, we speculated that the improvement in cell shape could be attributable to the effect of internalized enzyme. Although it cannot be expected that a single therapeutic approach would solve all the problems connected with PD, it could be assumed that a finite amount of prolidase constantly provided to fibroblasts with microspheres could be able to restore, at least partially, the cells' capacity to degrade undigested dipeptides X-Pro retained in the cells. On the basis of recent published data (Forlino et al 2002), it would seem that these dipeptides are responsible for the activation of necrosis-like events in PD cells.

Conclusions

The evaluation of different additives in the preparation of prolidase-loaded PLGA microspheres led to the conclusion that prolonged release of the active enzyme was achieved by preparing the microspheres in the presence of citrate buffer in the aqueous external phase of the double emulsion.

The system described in this study might provide a tool for specific treatment of prolidase deficiency. In fact, the experiments demonstrated that the system could carry amounts of active prolidase into fibroblasts, thus diminishing the accumulation of dipeptides X-Pro in the cells. It is this accumulation that is thought to be responsible for

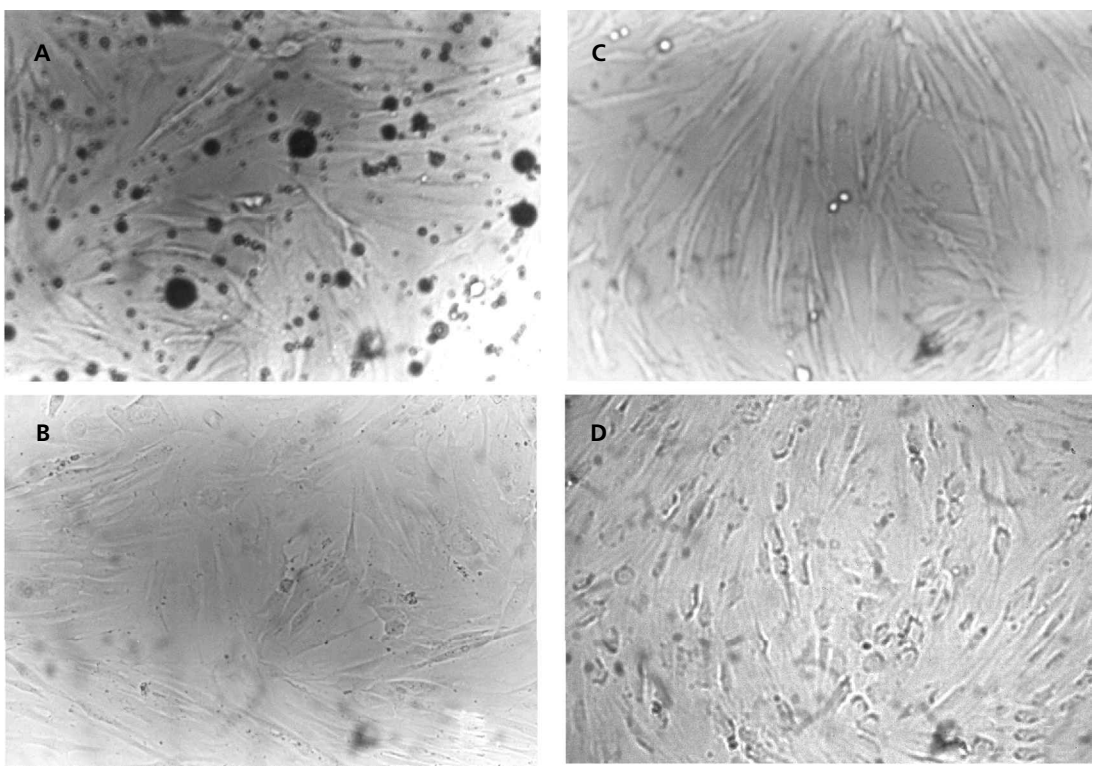

Figure 4 Contrast-phase light microscopy images of fibroblasts from a PD patient immediately after addition of microencapsulated prolidase (A) and after a 3-day incubation with microspheres encapsulating or not prolidase (C and D, respectively). B. Photograph of fibroblasts from a control.

the development of the clinical manifestations in man. Additional studies are necessary to understand the potential benefits of this practice. Nevertheless, in the absence of any alternative remedy for this disease, the procedure presented could be a possible candidate for the future development of a therapeutic approach to prolidase deficiency. The results seemed to indicate that it could be a reasonable way of improving cell morphology; whether this also implies an improvement of cell function and therefore of the clinical condition of patients is an important issue that needs to be answered.

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