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Research Article

Chitotriosidase and gene therapy for fungal infections

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Abstract. Chitotriosidase secreted by activated human macrophages has been implicated in the defence against chitin-bearing pathogens. The antifungal properties of human chitotriosidase were investigated here following retroviral vector-mediated gene transfer of the open reading frame of the chitotriosidase gene into Chinese hamster ovary cells. A chitinase assay confirmed that the engineered cells secreted recombinant chitotriosidase constitutively. Two dimensional gel electrophoresis and western blotting indicated that the recombinant protein is the major, chitin-binding, fifty kilodalton isoform. Culture medium conditioned by the transduced cells inhibited growth of isolates of *Aspergillus niger*, *Candida albicans* and *Cryptococcus neoformans*. Furthermore, longevity was significantly increased in a mouse model of cryptococcosis when cells transduced with the chitotriosidase gene and encapsulated in alginate microspheres were implanted subcutaneously in the animals. Engraftment of microcapsules containing cells transduced with the chitotriosidase gene has the potential to combat infections caused by chitinous pathogens through the prolonged delivery of recombinant chitotriosidase.

Keywords. Recombinant chitinase, antifungal agent, cell microencapsulation, gene therapy.

Introduction

Morbidity and mortality from invasive fungal infections have increased dramatically over the past two decades, especially in immunocompromised hosts. Antifungal drugs currently marketed for human use are of suboptimal efficacy or toxic, most targeting the ergosterol biosynthetic pathway. Exploration of new antifungal drug targets has resulted in the marketing of only one new class of compound, the echinocandins, which inhibit glucan synthase and hence cell wall synthesis.

Other components of pathogenic organisms, such as chitin, a linear polymer of β 1, 4-linked *N*-acetylglucosamine, are potentially attractive drug targets. Chitin is synthesized and incorporated as a structural component or as part of a protective coat by fungi, protozoa and nematodes [1], but not by humans. However, extensive evaluation of compounds such as Nikkomycin Z, a potent competitive inhibitor of

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fungal chitin synthase, which is active against some fungi *in vitro* [2, 3], has not resulted in a commercially viable drug.

An alternative approach to antifungal therapy, especially in the presence of prolonged immune suppression, is to enhance the immune system. Chitin turnover is affected by endogenous chitinases, which are members of the glycosylhydrolase family 18. These enzymes are ubiquitous in nature, including species that lack chitin. On the basis of the known antifungal properties of chitinases in plants [4], it has been suggested that chitinases in organisms that do not produce chitin play a role in the degradation of chitin-bearing pathogens. In humans, the chitinase, chitotriosidase, is produced in immune responsive phagocytes, namely activated macrophages and polymorphonuclear neutrophils [5–7]. It is encoded by the *CHIT1* gene located on chromosome 1q32, and is highly homologous with chitinases found in other species [8]. Two human isoforms are synthesised by alternative splicing of RNA or proteolytic processing, and possess functional chitinolytic domains. The major isoform is secreted, has a molecular mass of 50 kDa and a heterogeneous p*I* ranging between 5.0 and 7.2. The C-terminal domain of the 50 kDa isoform permits the enzyme to bind to chitin [9, 10], and crystal structure studies predict that it acts as an exochitinase [11].

Although a physiological role for chitinases in mammals has not been clearly identified, there is increasing evidence that they have fungicidal properties. For example, elevated levels of chitinases have been reported in guinea pig blood following systemic infection with *Aspergillus fumigatus* [12]. More recently, a recombinant form of the human enzyme was shown to hydrolyse artificial chitin analogues and inhibit fungal growth *in vitro* [13]. In related experiments *in vivo*, the enzyme prolonged survival of mice with systemic infections of candidiasis and aspergillosis, although this required daily injections to compensate for its short half-life [14]. Indirect evidence suggests that chitotriosidase also acts against other chitin-bearing pathogens. For example, individuals who have inherited an inactive chitotriosidase gene, resulting from a recessive null mutation, have an increased risk of infection with filiarial endoparasites in regions where these organisms are endemic [15]. Interestingly, elevated levels have been reported in a number of disease states, such as Gaucher's syndrome, β-thallassemia, atherosclerosis and neurological disorders, but its presence is thought to be related to increased macrophage activity associated with these disorders, rather than the aetiology of the disease [16]. To test the hypothesis that human chitotriosidase has antifungal properties *in vivo* if administered continuously, Chinese hamster ovary (CHO) cells were transfected with the human chitotriosidase gene cloned in a mammalian expression system. The genetically modified cells were encapsulated in alginate microspheres and implanted subcutaneously in mice infected with *Cryptococcus neoformans*. This alternative approach to gene therapy was explored in order to avoid permanent transmission of the gene construct to the host and to induce controllable, constitutive and prolonged release of the recombinant enzyme from the microcapsules *in vivo*.

Materials and methods

Mammalian expression system. The chitotriosidase cDNA was supplied by ResGen in a pcDNA3.1 vector (GeneStorm clone accession: U29615; Invitrogen Corp, CA, USA). This was sequenced at Westmead DNA, Millennium Institute, Westmead Hospital using external and internal primers (Table 1) to confirm insert homology with the open reading frame (ORF) of the human chitotriosidase mRNA sequence (GenBank accession NM_003465; and [17]). PCR amplification using primers PC1 and PC7 (Table 1) with high fidelity Herculase Enhanced DNA polymerase (Stratagene, CA, USA) generated a 1.6 kb ORF containing an upsteam Kozak sequence for stability and start codon, and restriction enzyme site specific sequences for directional subcloning into the pLXSN retroviral vector (Clontech, CA, USA) for stable transfection into mammalian cells. The recombinant clone, designated pLCSN, was transfected into competent JM109 cells and selected with ampicillin. Transformants were screened by PCR amplification using the PC1 and PC7 primers. Clones with the 1.6 kb insert, identified by agarose gel and sequencing analysis, were propagated and purified using an Endofree Plasmid Maxi Kit (Qiagen, Vic, Australia).

Cell culture. Murine NIH 3T3 fibroblast-derived packaging cell lines, ψ2 and PA317, were cultured in DMEM (ThermoElectron, Victoria, Australia) supplemented with 10% heat-inactivated foetal calf serum (FCS; JH Biosciences, Victoria, Australia). Chinese Hamster ovary fibroblasts (CHO) were propagated in CHO growth medium: HAMS/F12 medium (ThermoElectron) supplemented with 10 mM HEPES, 14 mM $NaHCO_3$ (Sigma Aldrich, New South Wales, Australia) and 10% FCS; or a modified Eagle's medium (TMEM; ThermoElectron) and 2% FCS, pH 7.4. Penicillin (50 i.u./ml) and streptomycin (50 μg/ml) were routinely used. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 in 75 cm^2 flasks, or in 3-tier cell factories (Nunc, Den-

Table 1. Primer sequences used for human chitotriosidase cDNA amplification and sequencing. The Kozak sequence used to confer stability to the start site of the open reading frame of the gene is underlined in primer PC1.

Primer	Sequence
PC1 external forward	5′-AAAAAGAATTCATCATGGTGCGGTCTGTGGCC -3’
PC7 external reverse	5′-AAAAACTCGAGTCATCAATTCCAGGTGCAGCATTTGCAG-3’
PC3 internal forward	5’-CTATGAAGTCTGCTCCTGGAAG-3’
PC4 internal reverse	5’-CTGCTTCAGATAGCTGACCTTG-3’

mark) to increase cell numbers for encapsulation (~ 8 x 10^7 cells/unit).

Human peripheral blood monocytes were isolated from whole blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, UK) following the manufacturer's instructions. Monocytes were cultured in RPMI-1640 medium (ThermoElectron) supplemented with 10% FCS, pH 7.4, and induced to differentiate with 10% heat-inactivated human AB serum (Sigma-Aldrich). The medium was replaced every 3 days (d) and monitored for chitinase activity over 27 d.

Transfections. The retroviral vectors, with and without the chitotriosidase gene (pLCSN and pLXSN, respectively) were introduced into CHO cells via sequential infections of the ecotropic (ψ2) and amphotropic (PA317) murine packaging cell lines [18, 19]. The ψ2 cells were transfected with 2 μg retroviral DNA/2.5 ml culture medium by lipofection using GeneJammer (Stratagene, USA) following the manufacturer's instructions. Virus-containing medium from ψ2 cells was used to infect the PA317 cells. Transduction of CHO cells was effected by a 26 hour (h) incubation in filtered virus-containing medium harvested from the transfected PA317 cells diluted with CHO growth medium without antibiotics (mixed 2 parts virions to 3 parts growth medium) and in the presence of 4 μg/ml polybrene (hexadimethrine bromide; Sigma-Aldrich). The transduced CHO cells were then subcultured and selected for 15–18 d in 1000 μg/ml neomycin (G418). Stable transformants were identified by chitinase assay of medium conditioned by these cells. Increased secretory activities were achieved by clonal selection, and optimal secretion rates and hydrolytic activities were realised in media maintained at pH 7.4.

Chitinase assay. Media conditioned by CHO+pLCSN or CHO+pLXSN cells were tested for chitinase activity using the method of Hollak et al. [20]. This measured the conversion of the chitin analog, 4–3 methylumbelliferyl β-D-N, N' N"-triacetylchitotrioside (220 μM; Sigma-Aldrich), to a fluorogenic product. Relative fluorescence units (RFU) were measured using a Fluorostar Optima microplate reader (BMG Lab Technologies, Victoria, Australia) fitted with 355 nm excitation and 460 nm emission filters. Enzyme activity was calculated from a standard curve of 4-methylumbelliferone (Sigma-Aldrich). Chitinase activity was calculated as μM hydrolysed substrate/ml conditioned medium or blood serum/min.

Antibody production. Epitope mapping of the 50 kDa chitin-binding isoform of the human chitotriosidase gene (GenBank accession: XP_001685) revealed an 18 mer peptide close to the C-terminus with a high predicted immunogenicity. A polyclonal rabbit antiserum immunoreactive against human chitotriosidase was raised against it. The 18-mer peptide was synthesized at Mimitopes (Victoria, Australia) and rabbit antiserum produced at the Institute of Medical and Veterinary Science (Adelaide, South Australia). The antiserum was affinity purified with the synthetic peptide covalently coupled to Thiopropyl-Sepharose 6B gel. Antibody specificity was determined by dot blot using synthetic peptide and conditioned media from activated human macrophages and transduced CHO cells as sources of antigen.

Expression analysis. Cell cultures were propagated in growth medium until confluence was reached then rinsed with and incubated in serum-free medium (~10^6 cells/10 ml CHO medium without serum) for a further 3–5 d before harvesting the conditioned medium. The medium was tested routinely for chitinase activity. Each flask of 10 ml conditioned medium was lyophilised, reconstituted in 1 ml MilliQ water and dialysed in 10 kDa MW cut-off tubing in two changes of 0.1 M NaCl for 24 h at 4 °C. Protein concentrations were measured using a DC protein assay kit (Bio-Rad, Ca, USA). Further purification was carried out by gel filtration (Sephadex 200HR, GE Healthcare Bio-Sciences Corp., NJ, USA) in 25 mM Tris buffer, pH 8.0. Eluted fractions were tested for chitinase activity and analysed by SDS PAGE and western blotting. Protein bands in the gels were visualised with Coomassie Blue. Proteins transferred to nitrocellulose membranes were probed with the affinity-purified chitotriosidase antibody (diluted 1:1000 in 3% skim

milk) for 1 h. Antibody binding was detected with a polyclonal antibody to rabbit IgG conjugated to horseradish peroxidase (diluted 1:3000; Transduction Laboratories, KY, USA) and visualised with a Renaissance Plus chemiluminescence kit (NEN Life Science Products, MA, USA). The M_r 50 kDa fractions showing positive chitinase activity were pooled and concentrated in an Amicon Ultra-15 centrifugal filter device (10 kDa MW cut-off; Millipore Corporation, MA, USA). Concentrated samples were focussed in an immobilised pH gradient (pH 3–10; BioRad Ready Strip IPG strips; 400 μg protein/strip) using a flatbed IPG Phor Isoelectric Focussing System (Amersham Pharmacia Biotech, CA, USA) after overnight incubation in rehydration buffer (8.72 M urea; 2.1 % Triton-X; 0.55 % BioRad IPG Buffer with carrier ampholytes; 20 mM DTT). The focussed strips were incubated in equilibration buffer (50 mM Tris, pH 6.8; 6 M urea; 30 % w/v glycerol; 2 % w/v SDS; 1 % w/v DTT) and electrophoresed in a 10 % polyacrylamide gel. Proteins were silver stained (Bio-Rad) or transferred to nitrocellulose membranes for immunoblotting with the chitotriosidase antiserum.

Antifungal susceptibility testing. Serum-free conditioned media harvested from confluent cell cultures (10 ml/10^6 cells) were partially purified to remove excess media by lyophilisation and dialysis only, as described above. Protein concentrations were measured and concentrations of the CHO+pLCSN and CHO+pLXSN conditioned media in each experiment were normalised by adjusting with PBS to the lowest protein concentration of one of the samples. Susceptibility was measured in isolates of *Candida albicans* (ATCC Strain 90028) and *Cryptococcus neoformans* (ATCC Strain 90112) using the standard broth microdilution method as described previously [21]. Susceptibility was tested in *Aspergillus niger* by measuring hyphal growth from spores. Spore suspensions in saline (~10 spores/well) within a cavity slide (Agar Scientific, UK) were mixed in equal parts with growth medium (10.4 g/L RPMI-1640; 0.165 M 3-(N-morpholino) propanesulfonic acid; 2 % glucose in MilliQ water; pH 7.0; tetracycline at 10 μg/ml final concentration) and partially-purified serum-free conditioned medium from CHO+pLCSN, diluted CHO+pLCSN medium (1:1) with PBS, or CHO+pLXSN. The cavity slides were sealed under coverslips with Vaseline and incubated at 30 °C in a humidified atmosphere for 18 h. Photographic images of hyphal growth were captured using a compound microscope with phase contrast optics (Leica Microsystems, Germany). Hyphal lengths (mm) were measured with a multi-scale map measurer and converted to μm using a stage micrometer (Electron Microscopy Sciences, UK).

Encapsulation of CHO cells. Transduced cells propagated in cell factories were harvested and encapsulated following the procedure of Lim and Sun [22] using a Research IE-50R Encapsulator (Inotech, Switzerland). Briefly, cells (5 x 10^6 cells/ml) were suspended in a 1.5 % solution of low viscosity sodium alginate (Sigma-Aldrich) and extruded by syringe pump through a 250 μm diameter nozzle at a flow rate of ~2 ml/min. Droplets were collected in 100 mM calcium chloride, incubated for 5 min to permit cross linking and coated successively in 0.0625 % poly-L-lysine and 0.15 % alginate for 10 min each. The microcapsules were washed, immersed in 55 mM sodium citrate (35 seconds), and transferred to CHO growth medium and incubated at 37 °C in 5 % CO_2. Chitinase activity was assayed in 3 ml medium conditioned by 100 microcapsules in 35 mm dishes. Media were changed every 2–3 d when capsules were transferred to fresh dishes and any broken capsules replaced with an intact capsule from the parent pool.

Engraftment of encapsulated cells in mice. The antifungal properties of the encapsulated CHO+pLCSN cells were tested in a mouse model for cryptococcosis. Protocols for the animal studies and the handling of genetically modified cells were approved by institutional Animal Ethics and Biosafety Committees and conformed to the guidelines of the Australian National Health and Medical Research Council and the Office of the Gene Technology Regulator. Female BALB/c mice (18–23 g) were sourced from Animal Resource Centre (Floreat Park, Western Australia). On the day of treatment, encapsulated CHO+pLCSN or CHO+pLXSN cells were loaded into 1 ml syringes (approx 1000 capsules in 0.4 ml medium) fitted with a 16-gauge needle and injected subcutaneously in the dorsal neck region of each mouse. The microcapsule suspension containing CHO+pLCSN cells were implanted into the experimental group of mice; while the control group received microcapsules with the CHO+pLXSN cells.

Survival and fungal burden studies. On either d 3 (survival protocol) or d 23 (fungal burden protocol) after microcapsule injection, 10^6 cryptoccocal cells (*C. neoformans* strain H99) were administered by tail vein injection as described previously [23]. Survival function studies were as previously described [24]. Each animal in the two groups was sacrificed when signs of illness were first evident (anorexia, weight loss, reduced activity, ruffled fur, cranial bulging) 6–21 d after infection. Any surviving mice were sacrificed at d 21. Blood was collected by cardiac puncture and lung and brain were removed and processed as described previously [23]. The fungal burdens in target organs

were expressed as mean colony forming units (CFU)/g tissue. Serum from blood samples were tested for chitinase activity using the chitinase assay. A different protocol was also used to compare fungal burdens in the target organs in the two groups. Here, the mice were sacrificed at one time point when the first animal showed signs of illness 5–6 d after infection. Blood and target organs were collected, and processed as above.

Statistical analysis. Data from the measurements of hyphae of *Aspergillus niger* in conditioned media from the CHO+pLCSN and CHO+pLXSN cells were analysed using STATISTICA Version 6 (StatSoft Inc, OK, USA; 2003) and Tukey's Honest Significant Difference Test. To determine the differences in the growth of *Candida albicans* and *Cryptococcus neoformans* in the two conditioned media, for each organism, regression analysis was performed on the optical density from each medium and on the combined data from both media using TableCurve 2D 5.0 (AISN Software). The residual sums of squares from the regression ANOVA were then compared following the procedure of Mead et al. [25]. Statistical analyses for the animal studies were undertaken using the Statistical Package for Social Sciences, version 15 (SPSS Inc, IL, USA). The survival data of mice engrafted with microcapsules with CHO+pLCSN and CHO+pLXSN cells were analysed by the Mantel-Cox log rank test. Fungal burdens (mean CFU/gm tissue) were analysed using general linear models. Level for statistical significance was defined by a p value of < 0.05 for all the tests.

Results

Mammalian expression system for a secreted human chitinase. Stable transformants of CHO cells transduced with the gene (CHO+pLCSN), or the empty vector (CHO+pLXSN, control), were generated after selection in the presence of 1000 μg/ml G418 in culture medium. Optimum secretion of chitotriosidase from CHO+pLCSN cells was obtained at a pH of 7.4. Chitinase activity of media conditioned by confluent cultures of high secreting clones ranged between 39.2–150.6 μM hydrolysed substrate/ml medium/minute for up to 5 d in culture. Subcultured cells remained viable, and the active enzyme was detected in the culture medium over many passages (> 20) indicating constitutive secretion. By comparison, chitinase activity was only found in media from activated human macrophages 6 d after activation. Activity peaked on d 17 (range 75.6–189.2 μM hydrolysed substrate/ml medium/minute) and then declined, being undetectable after d 25. Chitinase activity was not detected in media harvested from CHO+pLXSN cells or from human monocytes.

Protein analysis. To establish that the recombinant chitotriosidase was homologous with the 50 kDa isoform, conditioned medium was analysed by SDS-PAGE and 2-D electrophoresis, followed by immunoblotting. An antiserum raised to an epitope of the C-terminal chitin-binding domain detected a single band of the expected size in immunoblots of conditioned media from CHO+pLCSN cells (Fig. 1). No reactivity was detected in medium from CHO+pLXSN cells or untransfected CHO cells. Immunocytochemistry with the antiserum revealed fluorescent material localised in cytoplasmic vesicles in activated human macrophages and in CHO+pLCSN cells (not shown). Chitinase activity was detected exclusively in the eluted M_r 50 kDa fractions of the conditioned medium from CHO+pLCSN cells after purification by gel filtration (Fig. 2A). Two-dimensional gel electrophoresis of the fractions revealed that they contained three polypeptides with heterogeneous p*I*s between ~5.8–7.0 that reacted positively with the chitotriosidase antiserum in immunoblots (Figs. 2B, C).

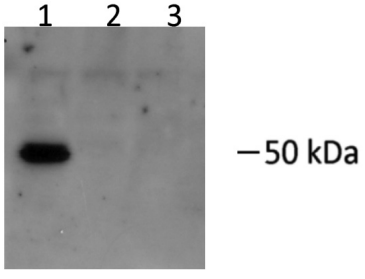

Figure 1. Immunoblot of conditioned medium harvested from transduced CHO cells. An antibody raised to the C-terminus of the major human chitotriosidase showed strong reactivity against a band with a M_r 50 kDa in CHO+pLCSN-conditioned medium (lane 1), but not with CHO+pLXSN-conditioned medium (lane 2) or medium harvested from untransfected CHO cells (lane 3).

Antifungal activity of conditioned medium. Chitinase activity of partially purified extracts of conditioned media from CHO+pLCSN ranged between 0.98 to 215.9 μM substrate hydrolysed/ml medium/min. As activity was not consistent between batches, and no activity was detected in the controls (CHO+pLXSN), protein concentrations were used to normalise the test samples before each experiment. Three fungal isolates were tested for susceptibility with the partially purified extracts.

Hyphal growth from *Aspergillus niger* spores was significantly inhibited following exposure to conditioned medium from CHO+pLCSN cells (chitinase

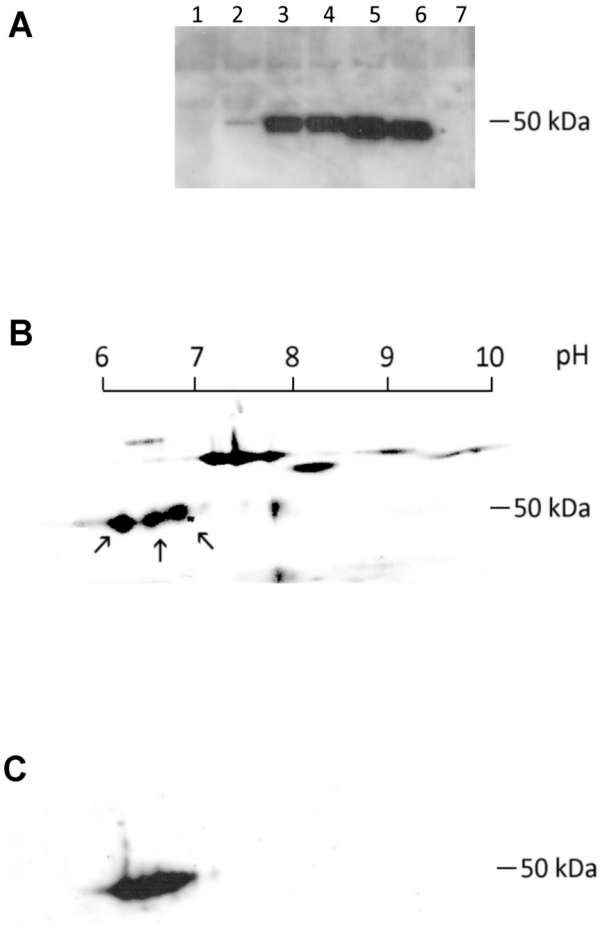

Figure 2. Recombinant chitotriosidase analysis. (*A*) Immunoblot of eluted fractions of CHO+pLCSN-conditioned medium after size exclusion liquid chromatography. Immunoreactivity with the antibody to human chitotriosidase was detected in the fractions run in lanes 2 to 6 as indicated by the detection of a band with M_r 50 kDa, which also corresponded to the fractions with chitinase activity. (*B* and *C*) Pooled 50 kDa fractions were concentrated and subjected to 2-D electrophoresis. (*B*) Proteins were separated in the first dimension using an immobilised gradient of pH 3–10, and in the second dimension using a 10% polyacrylamide gel. Three spots with M_r 50 kDa and p*I* between ~5.8 and 7 (arrows) were resolved in the gel by silver staining. (*C*) In an immunoblot of a duplicate gel, the chitotriosidase antibody reacted with proteins corresponding to the 3 spots (arrows in B) at 50 kDa.

activity 42.7 μM substrate hydrolysed/ml conditioned medium/min) at a protein concentration of 0.55 and 1.1 mg/ml, when compared with the controls CHO+pLXSN-conditioned medium with a protein concentration of 1.1 mg/ml and no chitinase activity ($p < 0.001$; Fig. 3A).

Similarly, comparison of regression analyses showed significant differences between the relationships of optical density and concentration of CHO+pLCSN-conditioned medium in microbroth assays using *Candida albicans* and *Cryptococcus neoformans*. Conditioned media from the CHO+pLXSN cells showed no significant inhibitory effect on cell growth in the

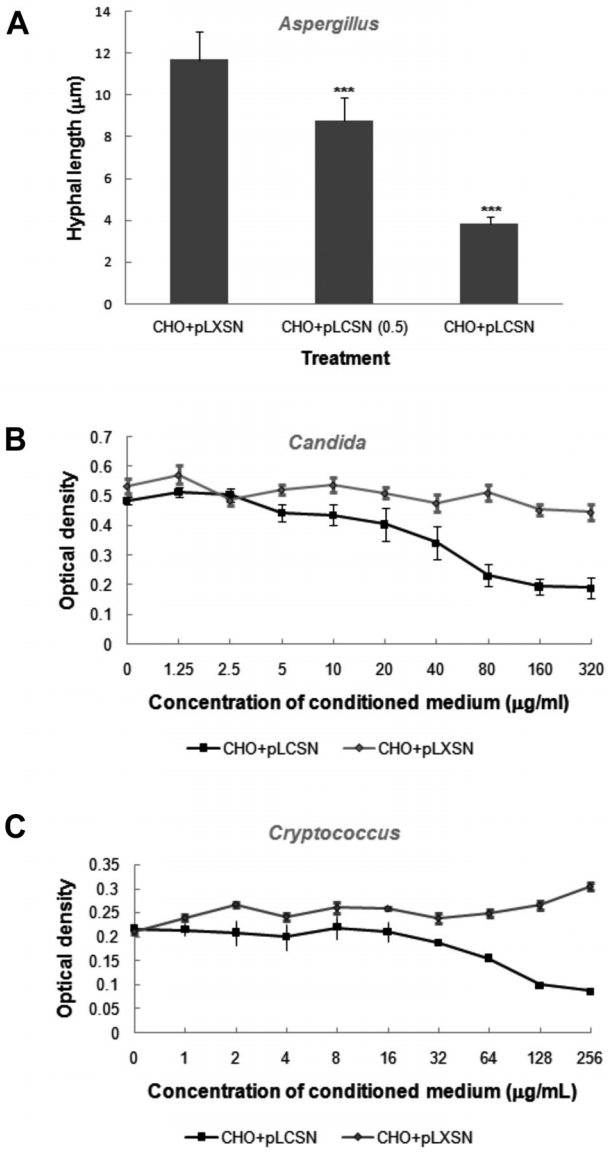

Figure 3. Antifungal properties of conditioned media *in vitro*. (*A*) Inhibition of hyphal growth from *Aspergillus niger* spores after treatment with conditioned media harvested from transformed cells with the chitotriosidase gene (CHO+pLCSN). Growth of hyphae (length in μm) was reduced significantly in CHO+pLCSN-conditioned medium at protein concentrations of 1.1 mg protein/ml, and at 550 μg protein/ml (CHO+pLCSN 0.5) when compared with controls (CHO+pLXSN) at 1.1 mg protein/ml concentration. Representative graph of three replicate experiments (***$p < 0.001$ compared to controls). (*B* and *C*) Susceptibility of yeasts to conditioned medium harvested from transformed cells with the chitotriosidase gene. Yeast cells were treated with serial dilutions of the conditioned media in microbroth assays. (*B*) A decrease in turbidity/optical density (OD) was detected in *Candida albicans* after exposure to CHO+pLCSN-conditioned medium (minimum inhibitory concentration $MIC_{50} = 80$ μg/ml). Cells incubated with CHO+pLXSN-conditioned medium were not significantly inhibited. Pooled data from three replicate experiments (*** $p < 0.001$ compared to controls). (*C*) A decrease in OD was detected in *Cryptococcus neoformans* after exposure to CHO+pLCSN-conditioned medium ($MIC_{50} = 128$ μg/ml). Cells incubated with CHO+pLXSN-conditioned medium were not inhibited. Pooled data from three replicate experiments (***$p < 0.001$ compared to controls).

Table 2. Inverse relationship of chitinase activity in blood serum (μM substrate hydrolysed/ml serum/min) and colony forming units (CFU)/gm target tissue in mice engrafted with encapsulated cells transduced with the chitotriosidase gene (CHO+pLCSN) and infected with *Cryptococcus neoformans.*

Mouse no.:	Days animal survived:	Chitinase activity (μM/ml/min):	CFU/gm lung tissue (x 10^7):	CFU/gm brain tissue (x10^8):
1	21	72.2	0	0
2	21	19.9	5.6	0
3	11	26.7	0.17	1
4	8	2.8	3.9	3.1

yeast isolates whilst, in comparison, fungal growth steadily declined as the concentration of medium conditioned with CHO+pLCSN was increased ($p < 0.001$; Figs. 3B, C). The minimum inhibitory concentration MIC_{50} for *Candida albicans* was 80 μg/ml CHO+pLCSN-conditioned medium. The chitinase activities of the five samples tested ranged between 15.3–215.9 μM substrate hydrolysed/ml conditioned medium/min, and all showed similar results. The MIC_{50} for *Cryptococcus neoformans* was 128 μg/ml with CHO+pLCSN-conditioned medium and a chitinase activity of 1 μM substrate hydrolysed/ml conditioned medium/min.

Chitinase activity of microcapsule secretions. Encapsulated CHO+pLCSN cells (mean microsphere diameter of 741 ± SE 5.26 μm) constitutively secreted the recombinant enzyme into their surrounding medium at a mean rate of 17.1 ± SE 4 μM/ml medium/min/100 capsules and a range of 2.3–39.9 μM/ml medium/min/100 capsules over 24 d (Fig. 4). As 50% of the culture media was replaced with fresh medium every 2–3 d the data indicated that the chitotriosidase secretion rate increased with time. Recombinant enzyme was continuously released from the capsules and retained chitinase activity for more than 50 d of culture (data not shown). No chitinase activity was detected in medium conditioned by encapsulated CHO+pLXSN cells over the same time period.

Antifungal activity of encapsulated cells *in vivo.* The efficacy of encapsulated cells releasing recombinant chitotriosidase as an antifungal agent was tested in mice infected with *Cryptococcus neoformans.* In order to determine its effect on longevity, a survival function protocol was employed. Microcapsules were injected 3 d before infection and animals monitored for 21 d after infection (26 mice/treatment group in three replicate experiments). Each mouse was culled when it showed debilitating symptoms of infection; usually apparent 6 d after the tail vein injection of *Cryptococcus*, or on day 21 if no illness was evident by the end of the experiment. The median survival time for the animals with encapsulated CHO+pLCSN cells was

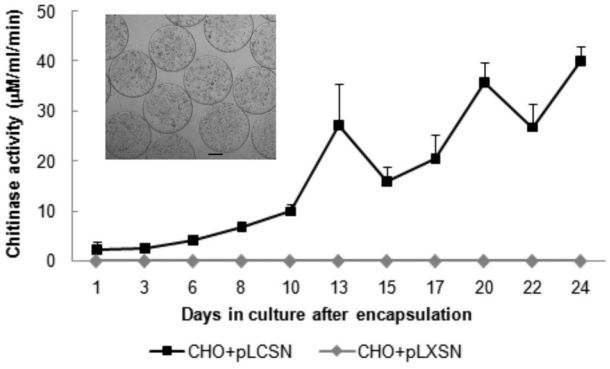

Figure 4. Chitinase activity measured in the microcapsule conditioned medium over 24 d. Encapsulated CHO+pLCSN cells constitutively secreted the recombinant enzyme, with chitinase activity of the medium increasing after 10 d incubation. The surges of chitinase secretion over time correspond to changes of culture medium every 2–3 d. Chitinase activity was not detected in medium harvested from CHO+pLXSN microcapsules (pooled data from two encapsulation preparations). Inset: Phase contrast micrograph of microcapsules containing CHO+pLCSN cells. Magnification bar represents 250 μm.

one day longer than controls with encapsulated CHO+pLXSN cells ($p=0.011$; Fig. 5). As most of the animals had succumbed to the fungal infection when culled, no significant differences were seen in mean fungal burdens in the target organs in the experimental mice compared with the controls. Chitinase activity, however, was detected in a small number of the blood samples that had been routinely taken from animals engrafted with encapsulated CHO+pLCSN cells. A decreased fungal load (CFU/gm) was observed in both brain and lung tissue in some mice ($n = 4$), which correlated with an increase in chitinase activity in the blood (Table 2). Two of the four mice had no infection in brain tissue and survived the 21 day period of the experiment, the data being consistent with an inverse relationship between blood chitinase values and CFU/gm tissue.

To measure fungal loads in target organs before morbidity was evident, a fungal burden protocol was used (16 mice/treatment group in two experiments). Here, the brains and lungs of all animals were harvested after culling on the same day when infection first became manifest (ruffled coat) in one mouse.

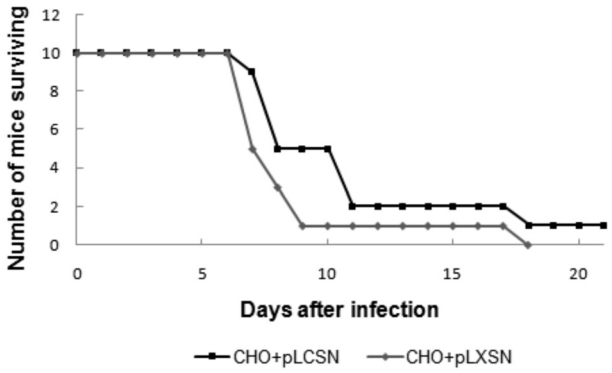

Figure 5. Survival rate in mice infected with *Cryptococcus neoformans* was significantly increased after engraftment with microcapsules containing CHO cells transduced with the chitotriosidase gene (CHO+pLCSN) compared with controls (CHO+pLXSN). Data from 3 experiments performed over a 21 d period (*$p < 0.05$).

Since the results of the survival studies suggested that blood chitinase activity increased with time after capsule engraftment (refer to Table 2), the mice were infected 23 d after injection of capsules. Mice were culled on d 6 after tail vein inoculation of *Cryptococcus* in the first trial, and on d 5 in the second. Overall there was a reduction in brain fungal burden in mice with the capsules containing CHO+pLCSN cells compared with control mice (mean CFU/gm tissue in mice engrafted with CHO+pLCSN cells vs. mice engrafted with CHO+pLXSN cells was $1.097 \times 10^8 \pm$ SE 0.05 vs. $1.23 \times 10^8 \pm$ SE 0.05 in the first trail; and $1.279 \times 10^8 \pm$ SE 0.075 vs. $1.354 \times 10^8 \pm$ SE 0.069 in the second trial; Fig. 6A) but the difference was not significant. There was also a tendency for reduced fungal load in lung tissue in the 6 d trial only in mice with encapsulated CHO+pLCSN cells when compared with controls (mean CFU/gm tissue in mice engrafted with CHO+pLCSN cells vs. mice engrafted with CHO+pLXSN cells was $4.975 \times 10^5 \pm$ SE 0.59 vs. $6.97 \times 10^5 \pm$ SE 0.75; Fig. 6B); however, this just failed statistical significance ($p = 0.056$). Chitinase activity was not detected in any of the blood samples collected.

Discussion

The identification and cloning of human chitotriosidase gene that encodes the human chitinase [5, 17] presented an opportunity to extend our understanding of its antifungal properties *in vitro* and *in vivo* and to investigate a novel gene therapy approach to antifungal treatment in mice infected with *Cryptococcus neoformans,* a cause of fatal meningoencephalitis and disseminated infection in humans.

CHO cells were transduced with the human chitotriosidase gene in a mammalian expression system and secreted recombinant enzyme at levels comparable to that of activated human macrophages in culture, and continued to do so over numerous passages. The molecular identity of the secreted protein was confirmed as the major secreted chitin-binding isoform of human chitotriosidase by 2-D gel electrophoresis and immunoblotting with an antibody raised against an epitope mapping to the C-terminus of the 50 kDa isoform. Three 50 kDa spots were resolved by isoelectric focussing. This heterogeneous nature of the chitotriosidase p*I* is consistent with previous reports

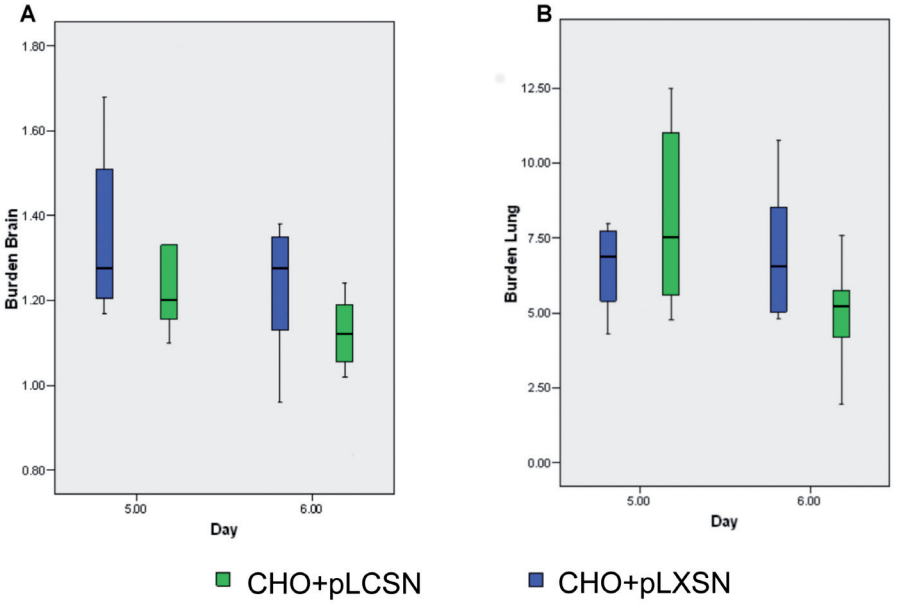

Figure 6. Comparison of fungal burdens in target organs of mice infected with *Cryptococcus neoformans* after engraftment with microcapsules containing CHO cells transduced with the chitotriosidase gene (CHO+pLCSN) or without the gene (CHO+pLXSN). Data from mice sacrificed at one time point on either d 5 or d 6 in the 2 trials. (A) CFU $x10^8$ / gm brain tissue. (B) CFU $x10^5$ / gm lung tissue.

and is thought to be related to variations in sialic acid content within its glycan composition [6, 9].

Culture medium conditioned by CHO+pLCSN cells showed antifungal activity towards filamentous fungi and yeasts *in vitro*. The mould, *Aspergillus niger*, a pathogen infecting both mammals and plants, showed a statistically significant reduction in hyphal length when treated with conditioned medium from CHO+pLCSN cells compared with controls. The yeasts, *Candida albicans* and *Cryptococcus neoformans* showed susceptibility with decreased growth, in conditioned medium from CHO+pLCSN cells but not in CHO+pLXSN-conditioned medium.

The transduced cells were also examined for their antifungal activity *in vivo* following encapsulation and engraftment into an animal model for cryptococcosis. Similar gene therapy approaches have been assessed for the delivery of recombinant proteins for treatment of a number of other medical conditions including diabetes, hypopituitarism, haemophilia and lysosomal-storage diseases [26, 27]. The purpose of cell encapsulation in an immunoneutral alginate matrix is to protect the genetically modified cells from dissemination and immune responses of the host. The pore size of the alginate matrix acts as a semi-permeable membrane, preventing host cells and large immunoglobulins from reaching the genetically modified cells in the capsule, whilst allowing the release of the smaller recombinant enzyme molecules into the host tissues. It was also considered that subcutaneous implantation of the capsules would provide an effective delivery system, whilst remaining accessible should removal, augmentation or replacement be necessary.

In the present study, the encapsulated CHO+pLCSN cells constitutively secreted active chitotriosidase *in vitro* for over 50 d. The evidence for continued secretion *in vivo* is less clear. Certainly, infected animals injected with CHO+pLCSN capsules showed a statistically significant increase in longevity when compared with controls engrafted with CHO+pLXSN capsules. The lack of significant differences in fungal burdens of the lungs and brains between the two groups was considered to be due, in part, to the nature of the protocol used, in which each mouse in both groups was culled when infection was manifest and the animal was close to death. However, chitinase activity was detected in a small number of experimental blood samples. In these cases, we noted that levels of chitinase activity were not only inversely related to the fungal burdens of the target organs, but positively correlated with improved survival in these animals.

A different protocol was designed for quantifying changes in fungal burden. The colony forming units of *Cryptococcus*/gm target organs in the two groups of mice were compared at the same time point after infection, all animals being culled at the first signs of illness in one individual. Overall there were lower levels of *Cryptococcus* colonies in the brains of animals engrafted with CHO+pLCSN capsules when compared with controls in the two experiments, but these were not statistically significant. Similarly, the mean fungal burden in lungs was reduced in treated mice in one out of two trials, but again was not significant. The observed reduction in fungal burden in the target organs suggest that experiments including larger numbers of animals would provide a more definite outcome, as marginal results are more likely to occur if the sample size is small. The data from all the animal experiments also suggest that the variations in responses of the treated mice were due, in part, to the failure of some capsules to secrete sufficient enzyme *in vivo*. The fact that we were unable to detect chitinase in the blood of most animals supports this suggestion. Clearly, other encapsulation technologies need to be explored to enhance transduced cell and microcapsule function and promote chitotriosidase delivery *in vivo*.

Others have reported that a recombinant chitotriosidase, administered by injection to mice infected with *Candida* or *Aspergillus,* disappeared from the circulation within 1 h [14]. Furthermore, its half-life in tissues was 3 h, necessitating reapplications to enhance survival. Certainly, a reliable, continuous supply of chitotriosidase to the circulation would be a more effective delivery system, and a gene-based approach, perhaps employing aspects of the encapsulated chitinase-secreting cell model described here, would provide an improved therapeutic effect.

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