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ABSTRACT

Macrophage Migration Inhibitory Factor (MIF) is a human cytokine that is well known for its role in modulating macrophage function. It has also been shown to cause smooth muscle cell death. A homolog of the MIF protein (BmMIF) has been found in *Brugia malayi*, one of the etiological agents of lymphatic filariasis. BmMIF shares a significant portion of its sequence homology with human MIF. Infection by *Brugia malayi* leads to a consistent finding of lymphatic dilation with surrounding smooth muscle cell apoptosis. We hypothesize that *Brugia malayi* releases BmMIF as a mediator of host smooth muscle cell death in the infected lymphatics in order to expand the lumen beyond normal anatomical dimensions. Our goal is to express and purify recombinant BmMIF and to measure the protein’s effect on smooth muscle cells in culture.

INTRODUCTION

*Brugia malayi* is an etiological agent of lymphatic filariasis, a disease that is predominant in the tropics and affects more than 120 million individuals worldwide. Infection is spread through the bite of a mosquito containing L3 larvae. The L3 migrate through the blood and enter the lymphatics and molt into L4, which in turn molt into adult parasites which reside in the lumen of the lymphatics. The adult parasites produce microfilariae that migrate from the lymph back into the peripheral bloodstream. Lymphatic filariasis is characterized by different categories of physical manifestations. Individuals with lymphatic filariasis present with a wide range of clinical signs; and may be asymptomatic, filaeremic or afilaraemic, or have clinical lymphangiectasia including both acute and chronic forms of the disease. In addition to the development of lymphatic filariasis, lymphangietasia due to filarial parasites forms a risk factor for additional chronic conditions such as lymphedema and elephantitis. As well, there are various psychological disorders that develop from the social stigma associated with the disfiguring nature of the disease.

One consistency in cases of lymphatic filariasis is lymphatic dilation. Irregardless of the clinical manifestation, lymphatic dilation is observed in all patients prior to patency of infection. The dilation of the lymphatics is due to the distention of vessel walls caused by adult larvae. The most commonly affected nesting location in the lymphatic circulation is the inguinal lymph node. Dilation of the lymphatics has been shown to be due to factors released by the parasite, the factor most likely being an excretory-secretory (ES) product of the parasite. Furthermore, lymphangietasia is not limited to the precise location of the L4 nest, contributing to the theory that the causal agent of dilation is likely an excretory-secretory (ES) product of the parasite. Dilation of the lymphatics can be induced by infecting SCID mice with *Brugia malayi*, and reversed by either removing or killing the worms, indicating the independence of this process from the host immune response.
Macrophage Migration Inhibitory Factor is a known human cytokine and acts as an inhibitor of random macrophage migration and thus enhances the retention of macrophages in tissues leading to the development of inflammation. In addition to immunological functions, MIF has also been implicated for a role in smooth muscle cell death. Taylor et. al has demonstrated that a null mutation in the MIF protein of mice prevents the death of bladder smooth muscle cells both in vivo and in vitro in the case of partial bladder outlet obstruction. Following take down, mice rendered null for the MIF were found to have increased muscle mass of the bladder of up to 40% in relation to wild-type mice. Primary bladder smooth muscle cultures treated with recombinant MIF exhibited an increased proportion of apoptotic cells seen on flow cytometry. Nucleated muscle counts in the bladder were 22% lower in wild-type mice than in MIF knock outs, indicating a loss of smooth muscle associated with the presence of MIF. This study was the first of its kind to elucidate the role of MIF in smooth muscle cell death. The impact of the protein on muscle survival and apoptosis was previously unknown.

An ES product of Brugia malayi and Wuchereria bancrofti, another filarial parasite that causes lymphatic filariasis, has been identified that has significant sequence homology with human MIF. Both BmMIF and human MIF show similar catalytic, structural, and physical properties. While BmMIF is present in the ES products of all stages of the parasite lifecycle, its expression is enhanced in the adult stage, the stage at which lymphatic dilation is observed. Antibodies to BmMIF are high in uninfected individuals residing in endemic areas, indicating that immunity to lymphatic filariasis may be due to host recognition of BmMIF.

If the sequence homology of human MIF and BmMIF is reflected in similar activity, then we expect BmMIF to be the mediator of smooth muscle cell death surrounding lymphatic vessels in lymphatic filariasis. The hypothesis is tested by expressing and purifying recombinant BmMIF and plating the protein on smooth muscle cell cultures. Recombinant BmMIF was prepared with the use of a pEXP5-NT/TOPO plasmid cloning reaction and expression was enhanced with isopropyl β-D-1-thiogalactopyranoside (IPTG). Various experiments were performed to determine the best method of protein purification. Ultimately, purification was performed with a nickel affinity column. Smooth muscle cell cultures were prepared by plating isolated cells obtained from the aortas of green fluorescent protein (GFP) positive mice. GFP fluoresces when exposed to blue light and is a commonly used biosensor for measuring gene expression. The lytic affect of both recombinant BmMIF and adult Brugia malayi parasites was measured by placing either recombinant protein or adult parasites on confluent cultures of smooth muscle cells and comparing the number of live smooth muscle cells pre and post-addition. To assess the biological activity of purified recombinant BmMIF, a macrophage migration inhibitory assay was used.

METHODS

Recombinant BmMIF Purification

The initial recombinant BmMIF protein obtained had been cloned into a BL21 vector by Dr. TV Rajan. Chemical precipitation techniques were used to purify and isolate the protein of interest from an overnight culture of BL21. Differing volumes of 200-proof
ethanol were added to BL21 lysate solutions. Following incubation at 0°C the samples were spun down in a microcentrifuge and the pellet and supernatant were separated. Both the pellet and supernatant were resuspended in 1% SDS sample buffer and were analyzed on a 12.5% PAGE gel with SDS gel electrophoresis. Subsequent chemical precipitation was carried out with ammonium sulfate by a similar procedure.

Chemical precipitation was followed by column separation of the BL21 lysate with a DE-22 column. The pH of the lysate was adjusted to alkaline conditions with NaOH. The pH of the DE-22 beads was brought to alkaline conditions with phosphoric acid. The beads were loaded into the column and were washed with phosphate buffer. 1 mL of BL21 lysate loaded onto the column and the eluent was collected in 0.2 mL fractions. The fractions were analyzed with SDS-PAGE on a 12.5% gel.

A soluble – insoluble preparation was performed to determine the solubility of BmMIF in order to determine how to best proceed with protein purification. Cells obtained from an overnight culture of BL21 were spun down and the pellet was isolated. Lysis buffer was added to the pellet and the sample was freeze-thawed for three cycles in ice cold ethanol and a 42°C water bath. The sample was centrifuged to pellet insoluble protein and the supernatant was transferred to a separate tube. Both the supernatant and pellet were resuspended in SDS buffer and run on a 12.5% PAGE gel.

Expression and Purification of BmMIF in a pEXP5-NT/TOPO Vector

In order to enhance the ease of expression and purification, BmMIF was re-cloned into a pEXP5-NT-TOPO vector, which facilitates purification by Ni-affinity chromatography. (Figure 1.1) The cloning of BmMIF was performed by Dr. TV Rajan. The plasmid was analyzed with a miniprep procedure and then transformed into BL21. Plasmids were mixed with BL21, heat-shocked, and incubated in AMP medium in a 37°C shaker overnight. (Figure 1.2) The expression of the transformed plasmid was then enhanced by the addition of IPTG.

The newly expressed BmMIF was purified using a combination of nickel column separation and dialysis. The Ni-NTA column was washed with a series of buffers and lysed BL21 was loaded onto the column. The tagged protein was eluded with buffer E
(100mM NaH$_2$PO$_4$, 100mM Tris-Cl, 8M Urea, pH 4.53) in 20 uL aliquots. The samples containing the most protein as identified by running an SDS PAGE gel were pooled and dialyzed slowly against buffer E without denaturant. Hydrogen peroxide was added to the buffer to help refold the protein. The concentration of BmMIF in the dialyzed sample was quantified by comparing a known volume of sample to differing concentrations of lysozyme on an SDS page gel. The vector derived N-terminal tag containing the poly-his sequence was cleaved from the recombinant BmMIF using acTEV protease.

**Assessment of Recombinant BmMIF Activity on Smooth Muscle Cell Cultures**

Smooth muscle cell cultures were derived from the digestion of GFP positive mice aorta. In this particular strain of mice, GFP expression is driven by a smooth muscle cell specific promoter Following take-down in a CO$_2$ chamber, the heart and attached aorta were removed from the mice and placed in cold sterile PBS buffer. The surrounding tissue (fat and adventitia) was removed from the aorta under a dissecting microscope. The cleaned aortas were cut into fine pieces and added to a sterilized solution of 5mg collagenase A, and 1mg of elastase in 10mL of PBS. The aortas and enzyme mixture were placed in a shaker at 37 °C for one hour, followed by centrifugation at 1600rpm for 5 minutes. The supernatant was removed from the sample and the volume was brought up to 40mL with sterile fetal calf serum plus penstrip. The cells, maintained in a solution of DMEM, 10% FCS, and Penstrip, were added to a Petri dish and placed into an incubator until confluent. Smooth muscle cells were identified and characterized by light microscopy.

The medium was removed from the culture with a vacuum line and 10ml of sterile PBS was added to reduce the concentration of serum protein. A 1.5mL solution of trypsin in EDTA was added to the cells to disrupt plate binding. 2mL of DMEM medium was then added to the cells to kill trypsin. Cells were counted with a Hemacytometer and were subsequently serum-starved for 24 hours. 0, 10, 25, 50, and 100ng recombinant BmMIF/mL was added in to each well in duplicate. Cells were analyzed with a florescent scope to determine smooth muscle cell necrosis.

Additionally, smooth muscle cell cultures from aorta and adipose tissue were trypsinized and frozen in DMSO. About one week prior to the arrival of Brugia malayi adult worms, an ampule of frozen cells of each cell line was thawed. Two days prior to the arrival of worms, the cells in the ampules were trypsinized and two wells of each smooth muscle cell type were plated on to 6-well culture plates. Approximately 4 adult worms, both male and female, were plated in one of the wells of each cell type. After five days the media containing worms was removed and the cells were incubated with trypsin until they were mobilized off of the plate. The number of living smooth muscle cells in each culture plated with adult worms was compared to the number of live cells in control wells.

**Macrophage Migration Inhibitory Assay**

Macrophages of cell lines RAW and J (obtained from ATCC) were grown up in cluster dishes. Once cells reached confluency, the cells were mobilized off of the wells and spun
down. The cells were pipetted into hematocrit tubes and spun for 5 minutes. The hematocrit tubes were broken at the cell – serum interface and the ends containing settled macrophages were adhered with silicon gel at the closed end to the center of the well of a six well cluster dish. Three wells were prepared for each cell line for a total of six wells. Media containing recombinant BmMIF and media containing recombinant protein PigR were added individually to one well of each cell line. Media was added to the last set of wells to serve as a control. The dish was observed for a period of a few days following set-up and the distance of macrophage migration out of the end of the capillary tube was observed and recorded.

RESULTS

Recombinant BmMIF Purification

Attempts at chemical precipitation were unsuccessful. In both ethanol and ammonium sulfate precipitations, BmMIF was not significantly purified from other bacterial proteins. From these findings it was concluded that chemical precipitation is an inefficient mode of BmMIF purification. (Figure 2)

![Figure 2.1 & 2.2: PAGE gels of chemical precipitation with EtOH and ammonium sulfate. Lanes 5 (60% EtOH precipitation pellet) and 7 (80% EtOH precipitation pellet) of the EtOH purification gels show an almost identical banding pattern to the unprecipitated BL21 lysate in lane 2. Lane 3 (40% EtOH precipitation pellet) shows one strong high molecular weight band, but this is too large to correspond to the BmMIF protein. MIF has a molecular weight of approximately 13Kd. The band may however represent a BmMIF dimer. Lanes 4, 6, and 8 contain very little protein, indicating that most proteins were isolated in the pellets. Lanes 3 – 6 of the ammonium sulfate precipitation gel show no significant difference in banding pattern or density from the unpurified BL21 lysate.

The DE-22 anion exchange column proved to be an inadequate method to purify BmMIF as well. The protein eluted as expected with the phosphate buffer washes, but was accompanied by an abundance of other bacterial proteins. Repeated trials of De-22
purification were unsuccessful and the results mirrored those previously described. (Figure 3)

**Figure 3:** PAGE gel of DE-22 column As indicated by the presence of bands in the flow through lanes 4 and 5, not all proteins were fully bound to the column. Perhaps the column was overloaded with sample. The majority of protein eluded in the first three elutions, E1-E3. No significant difference in banding pattern was found between the elutions and the BL21 lysate.

**Expression and Purification of BmMIF in a pEXP5-NT-TOPO Vector**

PCR followed by plasmid insertion was successful with the miniprep procedure identifying a clean band at the expected molecular size of plasmid plus DNA sequence. SDS page analysis of a BL21 culture following plasmid transformation revealed a protein of the expect molecular weight for the *Bm*MIF protein plus BL21. (Figures 4.1 and 4.2)

The use of a nickel column facilitated the isolation of *Bm*MIF from associated bacterial proteins. The tagged protein had an affinity towards the nickel beads, and eluted during late buffer washes. Dialysis became necessary to remove urea present in the elution buffers of the nickel column. Dialysis proved to be a timely procedure, and resulted in the loss of a large fraction of the purified *Bm*MIF. (Figure 5)

Based on comparison with known concentrations of lysozyme, the concentration of *Bm*MIF in solution was low. Addition of AcTEV protease to remove the N-terminal tag from the purified recombinant protein resulted in cleavage, although incomplete, of the N-terminal tag.
Figures 4.1 & 4.2: Miniprep results from generation of cDNA plasmid and PAGE gel results of plasmid transformation. The agarose gel prepared for the miniprep confirms the success of plasmid insertion. In the sample lane containing the plasmid reaction, distinct and clear bands can be seen that represent the cDNA plasmid, the supercoiled plasmid, and degraded RNA. The cDNA plasmid band is found at the expected molecular weight of the TOPO vector and incorporated PCR amplified BmMIF. SDS page results from plasmid transformation demonstrate in lane 4 a strong band representative of BL21 and the incorporated plasmid. This band appears slightly larger than that in lane 3, representing BL21 transformed with a smaller vector other than pEXP5-NT-TOPO.

Figure 5: Ni-affinity column chromatography facilitated clean purification of recombinant BmMIF. The absence of a BmMIF molecular weight band in the flow through sample indicates that all BmMIF was bound to the column. Bacterial proteins and dimerized BmMIF eluted in the flow through and in buffer C and D washes. Recombinant BmMIF eluted solely and independently in the buffer E washes.
Assessment of Recombinant BmMIF Activity on Smooth Muscle Cell Cultures

The affect of recombinant \textit{BmMIF} on smooth muscle cells was inconclusive. There appeared to be significant ballooning and clustering of cells corresponding to the addition of higher protein concentrations. Under the florescent scope, there appeared to be a slightly lower proportion of GFP positive smooth muscle cells (smooth muscle cells expressing the green florescent protein) in the wells with a higher addition of \textit{BmMIF}. The counting of dead and viable cells was not possible with the florescent scope, and would ideally require flow cytometry. The second batch of smooth muscle cell cultures were prepared from both mice aorta and uteri, however both cultures died before we were able to add any \textit{BmMIF}

From the smooth muscle cell cultures incubated with adult worms, it was found that the wells to which worms were added contained fewer cells than control wells to which no worms were added. In all three of the control wells, there were $1 \times 10^5$ cells per ml of live smooth muscle cells. One of the wells incubated with worms contained no live worms. The remaining two wells incubated with worms contained approximately $3 \times 10^4$ cells per ml each. A second trial of this experiment yielded very similar results. In the three wells to which no worms were added, the live cell number was $3 \times 10^5$ cells per ml. The three wells containing \textit{B. malayi} adults all contained less than $1 \times 10^4$ cells per ml.

Macrophage Migration Inhibitory Assay

Results from numerous trials have proven to be inconclusive, with no significant difference in cell migration observed between control and \textit{BmMIF} samples. In all wells, there was found to be a bubble of macrophages emerging from the tip of the hematocrit tubes. These bubbles were visualized under high power field of a light microscope, and were not measured but noted by presence and relative size. Both recombinant \textit{BmMIF} and PigR induced a similar pattern of cell migration. This finding is unexpected, and may indicate an error in assay method or analysis. PigR has no known affect on the motility of macrophages and it is expected to find a trail of cells emerging from the hematocrit tube. It was expected that \textit{BmMIF} inhibit the migration of macrophages, and that the movement of macrophages from the hematocrit tube into the surrounding medium would be retarded.

CONCLUSION

Despite the wide range of clinical manifestations, no optimal diagnostic technique exists for lymphatic filariasis. Compared to other parasitic diseases, details of the mechanism of \textit{Brugia malayi} infection are scarce. There exists a current lack of understanding of the pathway that leads to the development of lymphatic filariasis, and the maintenance of chronic versus acute infection. The function of \textit{Bm}-MIF and the mechanism of its formation are relatively unknown. By elucidating a function of \textit{Bm}-MIF-1 in host-parasite interaction, we can gain crucial knowledge that may assist in the development of novel therapies and vaccines targeting filarial parasites. An understanding of the role of \textit{Bm}-MIF in host-parasite interaction will also function to increase current understanding of the disease mechanism.
We have developed a method that once optimized, has the potential to result in expression and purification of BmMIF. The cloning of BmMIF into an NT/TOPO vector encoding an N-terminal polyhistidine tag facilitated and simplified the process of protein purification using Ni-affinity column chromatography. Since recombinant BmMIF was eluted from the Ni column in a buffer containing a high concentration of urea, it was necessary to dialyze the purified protein prior to measuring its affect on smooth muscle cells in culture. A significant proportion of BmMIF was lost during dialysis. Ideally the dialysis procedure could be modified to decrease protein loss, or the protein could be eluted from the column utilizing a lower concentration buffer. The presence of urea in buffer E may also possibly contribute to the modification of proper BmMIF folding.

We hypothesized that a protein produced by adult worms of Brugia malayi plays a role in the pathogenesis of lymphatic filariasis by causing smooth muscle cell death in the muscle layer surrounding the lymphatics, thus allowing the worms to multiply and the disease to propagate. The destruction of smooth muscle cells following incubation with adult B. malayi and the inconclusive findings of cell cultures incubated with purified recombinant BmMIF is suggestive of the following. Either BmMIF has the potential to cause smooth muscle cell death and the inconclusive results of incubated with recombinant protein are due to protein misfolding or inactivation that occurred during the purification procedure, or adult Brugia malayi parasites have the ability to kill smooth muscle cells independently of BmMIF.

Our results indicated that adult worms do indeed cause smooth muscle cell death. The worms are most likely to cause cell death by the release of a secretory-excretory product. However, his product may or may not be BmMIF. The inconclusive affect of purified recombinant BmMIF on smooth muscle cell culture is likely due to protein inactivation. The protein was exposed to numerous chemicals and reagents during purification that may have resulted in protein misfolding or perhaps even denaturation.

In order to confirm the hypothesis of protein inactivation, the activity of the purified BmMIF was measured using a macrophage migratory inhibition assay. The results of the assay, although inconclusive, suggest that the purified BmMIF may indeed be inactive. No inhibition of macrophage migration was observed for BmMIF in the assay, but measurements were relative and the procedure itself outdated.

To better assess the bioactivity profile of the purified BmMIF, we must develop a more valid and reliable assessment tool. Here we propose a modification and updating of the macrophage migratory inhibition assay. This assay was developed in the 1960s and has remained almost unchanged since this time. In the meanwhile, other biochemical methods and experimental techniques have been entirely changed, leaving the assay behind. The assay needs to be improved such that the measurement of macrophage migration is more quantitative, and the set-up simpler so there is no worry of disturbing set macrophages when medium is added to the wells.

We have preliminary evidence that B. malayi adult parasites kill smooth muscle cells in culture. As of yet, we have been unable to reproduce these effects with purified BmMIF. If the protein proves to be inactive, then the purification procedure will be modified to prevent future inactivation of the recombinant protein. Following the determination of the status of the purified protein, the effect of BmMIF on smooth muscle cell cultures will continue to be investigated. Further time and effort must be placed into developing an effective migration chamber assay in order to do so.
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