Mycobacterium Brumae Extract Fractions with Potential Immunotherapeutic Activity for Bladder Cancer

Mesane Kanseri için İmmünoterapötik Potansiyele Sahip Mycobacterium Brumae Ekstresi Fraksiyonları

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Abstract

Objective: Immunotherapy with intravesical Bacillus Calmette-Guérin (BCG) application is a gold standard treatment for high risk non-muscle invasive bladder cancer (NMIBC), despite its local and systemic side effects. We aimed to develop less toxic and more powerful therapeutic agents for the treatment of bladder cancer than live strain.

Materials and Methods: Non-pathogenic *Mycobacterium brumae* strains resembling BCG in immunostimulating and cytotoxic activities were used. The bacteria were sonicated after heat treatment. Samples prepared by aqueous solution and acetone were subjected to high performance liquid chromatography on reverse phase and strong ion exchange columns. Tumour necrosis factor-α (TNF-α) stimulating activities and the matrix-assisted laser desorption/ionization-mass spectroscopy findings of the samples were analysed.

Results: As a result, it was revealed that two components, m/z ratios around 1800 and another around m/z 3600 could be responsible for TNF-α stimulating activity.

Conclusion: These components have a potential to develop of new agents for NMIBC treatment.

Keywords: *Mycobacterium brumae*, non-muscle invasive bladder cancer, high performance liquid chromatography, matrix-assisted laser desorption/ionization-mass spectrometry, tumour necrosis factor-α

Öz

Amaç: Lokal ve sistemik yan etkilerine rağmen, yüksek riskli kasa invaziv olmayan mesane kanserinde (KİOMK) Bacillus Calmette-Guérin (BCG) uygulanması altın standart tedavidir. Biz mesane kanseri tedavisi için canlı suşlar yerine daha az toksik ve daha güçlü terapötik ajanlar geliştirmeyi hedefledik.

Gereç ve Yöntem: İmmünostimülan ve sitotoksik aktiviteleri BCG ile benzerlik gösteren patojen olmayan *Mycobacterium brumae* suşu kullanıldı. Bakteriler ısı muamelesinden sonra sonike edildi. Sulu çözeltiyle ve asetonla ekstre edilerek hazırlanmış örnekler ters faz ve kuvvetli iyon değiştirici kolonlar kullanılarak yüksek performanslı sıvı kromatografisi uygulandı. Örneklerin tümör nekroz faktörü-α (TNF-α) uyarıcı aktiviteleri ve matriks-yardımlı lazer desorpsiyon-iyonlaştırmalı-kütle spektrometri çıktıları incelendi.

Bulgular: Sonuç olarak, m/z oranları 1800 ve 3600 dolayındaki iki bileşenin TNF-α uyarıcı aktiviteden sorumlu olabileceği ortaya çıkarıldı.

Sonuç: Bu bileşenler KİOMK tedavisi için yeni ajanlar geliştirme potansiyeline sahiptir.

Anahtar Kelimeler: *Mycobacterium brumae*, kasa invaziv olmayan mesane kanseri, yüksek performanslı sıvı kromatografisi, matriks-yardımlı lazer desorpsiyon-iyonlaştırmalı kütle spektrometri, tümör nekroz faktörü-α

Introduction

Bladder cancer is the fourth most common cancer among men, and the 14th most common type among women (1). Non-muscle invasive bladder cancer (NMIBC) (which makes up 80% of the disease) has a low mortality rate, but a high recurrence rate (up to 70%) which requires attention with novel therapies. Immunotherapy with intravesical Bacillus Calmette-Guérin (BCG) application is a gold standard treatment for certain high risk NMIBC. International Bladder Cancer Group recommends this therapy for intermediate and high risk diseases (2,3,4,5). For high risk disease it is recommended to continue intravesical immunotherapy for 1-3 years (2,6).

It seems that mycobacteria stimulate the Toll-like receptors at the target cells to cause secretion of tumour necrosis factor-α (TNF-α) and several other cytokines. Antitumoural effects started by BCG include the interaction of several soluble and cellular immune system regulators, eventually destroying the tumour cells by an indirect immune cytotoxic action (7,8).

Although immunotherapy with BCG is an effective method, it has several local and systemic side effects such as cystitis, haematuria, lung infection, liver toxicity and sepsis (9) and protracted (maintenance) therapy period potentiates the risk of the side effects.

Research has been carried on to find less toxic and more potent immunotherapeutic agents. *Mycobacterium phlei* was found to be a promising strain for this purpose (10,11,12). It is shown that cell wall extracts of *M. phlei* (10,12) and *Mycobacterium smegmatis* (13) rich in proteins had immunostimulating activity. Our group searched the TNF-α stimulating activities of the cell wall extracts of 88 mycobacterium strains and found out that there are some more candidates for the treatment of bladder cancer (14). *Mycobacterium brumae* was among these strains. It is a non-pathogenic, rapid growing mycobacterium strain and the cell wall extract of which was as efficient as that of BCG for TNF-α stimulating activity. Its cytotoxic activity for human bladder cells measured by MTT test was found to be comparable to that of *M. phlei* (15). Recently, it was reported that *in vivo* antitumour activities of BCG and *M. brumae* were similar in tumour bearing mice (16).

It was previously reported that autoclaved BCG had no effect on tumour growth (17), but we obtained significant immunostimulating activity with heat treated *M. phlei* (12) and *M. brumae* (14) extracts although less efficient when compared to live bacteria. Recently, antitumour effect was also observed with gamma irradiated *M. brumae* cells although less efficiently (18). *M. brumae* is reported to be non-pathogenic for humans, but inactivated cells diminish the risk of inflammation completely. Therefore, it was of interest to fractionate the *M. brumae* cell wall extract by high performance liquid chromatography (HPLC) and try to determine the fractions responsible of the TNF-α stimulating activity.

Materials and Methods

Materials

M. brumae (ATCC 51384) cells were obtained from Salubris A.Ş., Turkey. Human monocyte cells (THP-1) were obtained from the Foot and Mouth Disease Institute, Cell and Virus Bank (HÜKÜK), Ankara, Turkey. Middlebrook 7H9 broth was from Difco, USA; ELISA plates and cell culture plates were from NUNC A/S, Denmark; TNF-α ELISA kits (KHC3011) were from BioSource (Invitrogen, USA). Löwenstein-Jensen culture media was from Salubris A.Ş., Turkey. Penicillin, streptomycin and RPMI-1640 medium were from Gibco (Gaithersburg, Maryland, USA). All the other chemicals were analytical grade.

Growth of *Mycobacteria*

M. brumae was grown as previously described (14). The cells were inoculated into Löwenstein-Jensen culture media supplemented with 10% oleic acid-albumin-dextrose complex and incubated at 37 °C. When colonies were visible, they were transferred into Middlebrook 7H9 broth culture media containing 0.2% glycerol; incubated in a rotary shaking incubator (Nüve, Turkey) at 37 °C for 1 week. The purity of the culture was checked with acid-fast staining.

Preparation of the Samples

M. brumae cell wall extracts were prepared with some modifications of the previous method (14). Cells were harvested by centrifugation at 1000 x g, washed twice with 50 mM potassium phosphate buffer, pH 7.4. The pellet was suspended in two volumes of the same phosphate buffer. The suspension was incubated at 95 °C for 10 minutes, sonicated for 2 min (at 40% amplitude, Sonics Vibra Cell VCX 750, USA), centrifuged for 20 minutes at 27000 x g. The supernatant was concentrated at rotatory evaporator (BUCHI R210, Swiss), dissolved in 50 mM potassium phosphate buffer, pH 7.4, and filtered through a filter with 0.45 mm mesh. This procedure for cell wall preparation did not disrupt the cell membrane as observed by microscopy. UV absorption spectra did not indicate the presence of DNA either.

Treatment of Monocytic Cell Lines with Mycobacterial Extracts

The monocytic cell line THP-1 was cultured in a humidified atmosphere of 5% CO_2 at 37 °C as previously described (14). The culture medium was RPMI-1640 containing heat-inactivated foetal calf serum (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL). 500000 cells/mL/well were dispensed in 24-well microtiter plates in the same medium. Cells were incubated with 200 nm of phorbol-12-myristate-13-acetate (PMA)/mL for 48 h at 37 °C. PMA-treated THP-1 cells were treated with mycobacterial extracts (5 µg sugar/mL). After 48 h of incubation, the culture supernatants of THP-1 cells were collected, centrifuged at 13000 x *g* and stored at -80 °C until used. Lipopolysaccharide was used as positive, and dilution buffer was used as negative control (19). Sugar content of the samples were measured by phenol sulphuric acid method with glucose as standard (20).

Measurement of Tumour Necrosis Factor-α Activity

ELISA for TNF-α was performed according to the manufacturer's instructions (Biosource, Invitrogen, USA). 50 µL of standard diluent buffer was added to the wells containing cell culture samples and 100 µl of the standard diluent buffer were added

to blank wells. 100 μL of standards, samples or controls were included to the appropriate microtiter wells. The plates were incubated for 2 hours at room temperature. Solution from wells was discarded and wells were washed 4 times. 100 μL of biotinylated anti-TNF-α solution was pipetted into each well except the chromogen blank(s), and mixed. The plates were incubated for 1 hour at room temperature. The solution was discarded and the wells were washed 4 times. 100 μL streptavidin-horseradish peroxidase working solution was added to each well except the chromogen blanks. The plates were incubated for 30 minutes at room temperature. The solution was discarded and the wells were washed 4 times. 100 μL of stabilized chromogen was added to each well. The plate was incubated for 30 minutes at room temperature in the dark. 100 μL of stop solution was added to each well. The measurements were done in triplicate. Absorbances were measured at 450 nm using Spectra Max M2 microplate reader (Molecular Devices; Canada) (19).

High Performance Liquid Chromatography Fractionation

HPLC separation of the samples were done with reverse phase chromatography on Shimadzu, semi-quantitative system, using C18 (Teknokroma Europa Peptide 120 C18 5 μm 25x0.7 cm) and strong cation exchange (SCX) (Teknokroma, Tracer Extrasil SCX 5 μm 25x0.7 cm) columns.

Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry

Alpha-cyano-4-hydroxycinnamic acid matrix (α-CHCA) was prepared in acetonitrile/water/trifluoroacetic acid (TFA) mixture (1:1:0.001, v/v/v). All the samples were mixed with the α-CHCA matrix solution at 1:10 (v/v) ratio by gentle stirring with a vortex mixer. A 1.0 μL portion of all the final solutions was directly spotted onto the matrix-assisted laser desorption/ionization (MALDI) sample target and dried at room temperature before analysis. MALDI-mass spectrometry (MALDI-MS) analyses of the samples were carried out in positive ion mode using a MALDI-time-of-flight-MS (MALDI-TOF-MS) (Applied Biosystems Voyager DE PRO, USA) with delayed extraction. Ions, formed by a pulsed UV laser beam (N_2 laser, λ=337 nm) at around 10^{-7} Torr, were accelerated with a 23 kV electrical potential. All spectra were the average of 50 shots and internally calibrated (21).

Statistical Analysis

ELISA results are presented as mean ± standard deviation. Statistical comparisons were made by Mann-Whitney U test. Statistical significance was assumed as p<0.05.

Results

Separation of the Cell Wall Extracts by Column Chromatography

Figure 1 shows the outline of the several chromatographic steps applied to the sample. Initially 500 μL of cell wall extract was applied to the C18 column. For the elution, water with 0.1%

TFA was applied with a flow rate of 0.9 mL/min for the first 50 minutes and after 50th minute elution was continued with 30% acetonitrile with a flow rate of 2 mL/min. Three peaks (C1 at 15-19 min, C2 at 19-22 min, and C3 at 70-80 min) were obtained by this procedure. The peaks were collected and evaporated separately and 500 μL of each were applied to SCX columns with a flow rate of 2 mL/min. Mobile phase was 5 mM potassium phosphate buffer, pH 3, containing 25% acetonitrile. Gradient elution was achieved by the same buffer containing 0.5 M NaCl. Sample C1 yielded 2 peaks (C1S1 at 5-10 min and C1S2 at 10-16 min). Sample C2 yielded 3 peaks (C2S1 at 7-10 min, C2S2 at 10-22 min and C2S3 at 22-30 min).

Sample C3 did not give a good resolution on SCX column. Eluates obtained between 3-14 min (C3S1), 14-25 min (C3S2) and 58-74 min (C3S3) were combined separately, dialysed, evaporated and each were applied onto C18 columns. On C18 column, C3S1 and C3S2 each yielded two peaks (C3S1C1 at 2-4 min, C3S1C2 at 4-6 min; C3S2C1 at 4-5 min, C3S2C3 at 5-6 min); and C3S3 yielded three peaks (C3S3C1 at 9-11 min, C3S3C2 at 11-13 min, and C3S3C3 at 40-52 min). Sample sizes of C3S1 and C3S3 were 500 μL and sample size of C3S2 was 1 mL. For these three separations, flow rates were 3 mL/min, mobile phase was water containing 0.1% TFA for the first 15 min and later, 30% acetonitrile.

Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry Profiles and Immunostimulating Activities of the Samples

MALDI-MS profiles of the cell wall extract and the samples obtained from the chromatography steps are at Figure 2. Table 1 summarizes the results of TNF-α stimulating activities of the samples and the notable MALDI-MS signals. All the samples contained several signals between m/z 1000-10000. Since protein content of some of the fractions were very low, sample volumes were calculated on the basis of sugar contents of the samples.
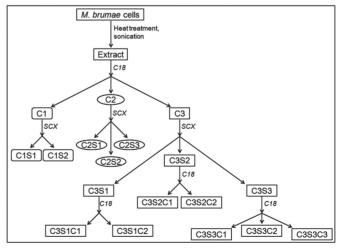

Figure 1. Diagram of the fractionation of the *Mycobacterium brumae* cell wall extract

TNF-α stimulating activity values of the cell wall extract and all the other fractions were comparable to each other except for C1S2 which had no activity at all and C2S1 which had very low activity. It is observed from the MALDI-MS spectra of Figure 2B that the fractions derived from peaks C1 and C2 show very weak signals between 1000-10000 range. The peaks derived from C3 had significantly higher immunostimulating activities as compared to the whole cell wall extract except for C3S2C1 and C3S3C3. The activities of these two peaks are comparable to the whole cell wall extract. The highest TNF-α stimulating activity was observed with the sample C3S3C1. This sample was rather pure and its main signal was at m/z 3660. Almost all the fractions derived from C3 had this MALDI-MS signal as the base signal, and most of them had significantly high immunostimulating activities as compared to the whole cell wall extract. The second signal of C3S3C1 with m/z 1830 had half the m/z ratio of the base signal, and it appeared only in the spectrum of C3S2C1 in addition to C3S3C1. The third signal in

the spectrum of C3S3C1 was rather a minor signal at around m/z 1290. Since the signal around m/z 1290 also appeared in the samples which had very low or no immunostimulating activity (C1S2 and C2S1), it is concluded that the component which has m/z ratio around 1290 does not contribute to the immunostimulating activity. From the spectra of all the samples it is concluded that the signals around 1800 Da and 3600 Da are responsible of the immunostimulating activity.

Discussion

In search for an alternative for BCG for the treatment of bladder carcinoma, we previously tested the immunostimulating activities of several mycobacteria strains and found out that some non-pathogenic strains can be candidates for the immunotherapy (13). Cytotoxic activities of these strains against human bladder tumour cells were formerly measured by MTT test (15) and found out that IC50 value for *M. brumae* was comparable to that of *M. phlei* which was another strain that can be used in bladder tumour treatment (10).
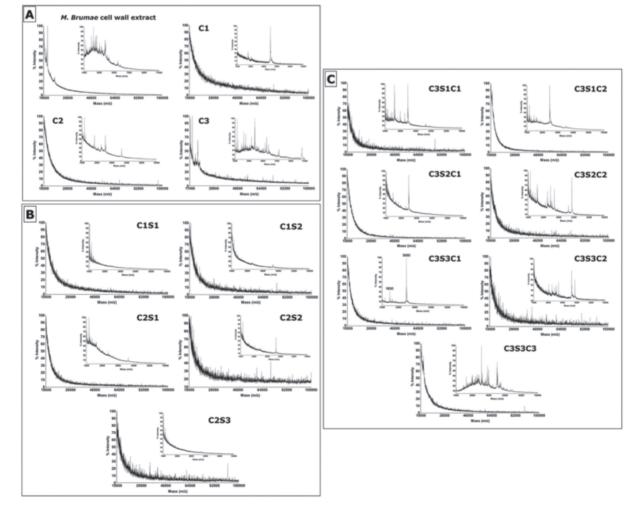

Figure 2. a) Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) spectra of *Mycobacterium brumae* cell wall extract and C18 fractions (C1, C2, C3), b) MALDI-MS spectra of strong cation exchange fractions of C1 (C1S1, C1S2) and C2 (C2S1, C2S2, C2S3), c) MALDI-MS spectra of C3S1C1, C3S1C2, C3S2C1, C3S2C2, C3S3C1, C3S3C2, C3S3C3

Table 1. Tumour necrosis factor-α stimulating activities and the matrix-assisted laser desorption/ionization-mass spectrometry signals of the samples*			
Samples	Sugar conc. (mg/mL)	TNF-α stimulating activity (pg/5 µg sugar)	Nominal mass of matrix-assisted laser desorption/ionization-mass spectrometry signals
Cell wall extract	15.26	105±19	1180, 2230, 2410, 2600, 3800, 3860, 10200, 10590, 12250, 12900
C1	3.82	96±36	3000, 5770
C2	3.45	134±47	1290, 2506, 3820, 5770
C3	2.96	70±10	1290, 2230, 2510, 3340, 3820, 5290, 5510, 7280, 12900, 16900
C1S1	2.40	102±27	1010, 1079, 1290
C1S2	1.02	0**	1080, 1290
C2S1	1.38	20±16**	1290, 1420, 2130, 3480, 5770
C2S2	1.74	156±56	1290, 5770
C2S3	0.31	130±16	1010, 1290
C3S1C1	0.12	178±13***	1080, 2090, 2110, 2125, 3660, 3670
C3S1C2	0.55	235±40***	1080, 1290, 3660, 3670
C3S2C1	0.54	130±27	1000, 1830, 3660, 3670
C3S2C2	0.04	228±26***	1080, 1290, 2090, 3300, 3660, 4120, 6180
C3S3C1	0.07	471±114***	1290, 1830, 3660
C3S3C2	0.12	196±23***	1080, 2890, 3820, 4120, 5770, 6180
C3S3C3	0.23	137±38	2130, 3800, 3900, 4470, 4590, 5530, 5580

*Negative control yielded zero absorbance and 1 µg/mL lipopolysaccharide yielded an absorbance comparable to C3S3C1, **Significantly lower than cell wall extract, $p<0.05$, ***Significantly higher than cell wall extract, $p<0.05$

In our former experiments with stepwise fractionation of the mycobacteria cells, we observed that the immunostimulating activity was mainly in the cell wall. Microscopic examination and the spectrophotometric measurements showed no nucleic acid involvement in the active fragments. Since whole cell extracts and the cell wall extracts yielded similar results, in the screening experiments with 88 strains, whole cell extract was preferred.

Since some of the samples had very low protein content for quantification, and since cell wall is rich in glycans (22), instead of protein content, sugar content of the samples were used for the standardization of the contents in each sample. Phenol sulphuric acid method (20) applied to microplates was used for the determination of the sugar content. Glucose and galactose gave similar standard curves and glucose was selected as standard.

The structures responsible for the observed activity are water soluble. In preliminary experiments, when the sample was extracted with acetone, the immunostimulating activity had reduced substantially and the signal around m/z 1800 was diminished.

In a previous study, Wang et al. (23) obtained 60-90 kDa glycans with anti-tumour activity from *Mycobacterium bovis* which are "heat resistant and not soluble in ethanol and acetone" (23). In our previous studies with *M. phlei*, we also obtained water soluble structures of m/z 3808, 9207, 11533, 12460 and 21587 which were able to stimulate cytokine activation (12). The signal at m/z 1830 reported here for *M. brumae*, which was also soluble in aqueous medium and not extractable with acetone seem to be among the structures responsible of TNF-α stimulation. Our results are in accordance with our previous findings (14) and with the findings of Wang et al. (23). Molecular mass differences observed may be due to the strain differences or the differences in the extraction procedures applied. On the other hand, our findings are not in accordance with Chin et al. (24) who found out that an extract rich in DNA was responsible of the immunostimulating activity. Spectroscopic studies of our samples did not indicate the presence of nucleic acids.

Cancer immunotherapy is one of the major breakthroughs of cancer treatment of the last years. Agents that activate the immune system, vaccines and the immune checkpoint inhibitors are under intensive investigation. Several phase I and II studies are under way and some agents are approved by the Food and Drug Administration. Some of the reviews about this subject are found at (25,26,27). Although whole mycobacteria cells can be instilled only to bladder and can be used for the treatment of NMIBC, adjuvants prepared can be used through

other routes; and they may be used for treatment of some cancers other than NMIBC in the future.

Study Limitations

This work focused on the detection of the *M. brumae* cell wall fractions with potential immunotherapeutic activities for NMIBC. The next part of the work is addressed to whole proteomics studies in order to identify the structures which show TNF-α stimulating activity. *In vivo* effects of these structures on animal models will also be a subject of a further study. These points make up the limitations of the study.

Conclusion

In this work, immunotherapeutic activities of the *M. brumae* cell wall fraction were examined and the masses of glycopeptides and glycoproteins were screened using MALDI-TOF-MS. In conclusion, a water soluble fraction with nominal molecular masses around 1800 Da and 3600 Da obtained from the cell wall extracts of *M. brumae* contained TNF-α stimulating activities. These fractions were obtained repeatedly in different sets of experiments. The structures obtained have a potential for the development of new agents for the immunotherapy of NMIBC.

Ethics

Ethics Committee Approval: Approval is not required.
Informed Consent: Informed consent is not required.
Peer-review: Externally peer-reviewed.
Authorship Contributions
Design: H.Ö., N.L.A., Experiments: Z.E., E.B., Ö.Ç., Interpretation of MALDI-TOF-MS results: B.S., Literature Search: Z.E., E.B., H.Ö., Ö.Ç., B.S., N.L.A., Writing: N.L.A.
Conflict of Interest: Zeliha Ertürk received scholarship, Esra Büber, Haluk Özen and N. Leyla Açan received honorarium, N. Leyla Açan received travel grant from TÜBİTAK. The authors Zeliha Ertürk, Esra Büber, Haluk Özen and N. Leyla Açan have a patent on "*Mycobacterium brumae* cell wall extracts that can be used in therapy of superficial bladder cancer" with application no: TR 2011/001874.

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