

Effects of *Neisseria meningitidis* Infection in Tumor Glioblastoma Cell Line NG97: Respiratory Pathogen Inducing Apoptosis

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Abstract

Astrocytomas, or glioblastomas, are aggressive malignancies of the central nervous system which has poor prognosis, even when submitted to surgery, radiation and chemotherapy. Established permanent cell lines are valuable tools for the study of the glioma pathology, diagnostic and treatment. In this work, were analyzed the adhesion, induction of apoptosis and chemokines' expression of the bacterial pathogen *Neisseria meningitidis* in the astrocytome cell line NG97. The analysis of the adhesion and morphological alteration by optical microscopy showed significant alterations mediated by meningococci infection in NG97 cells. Scanning-electron microscopy assays demonstrated a decrease in the number of microvilli on the cell surface as well as the meningococcal adhesion to the cells matrix when compared with the non-infected NG97 cells. Cells infected with different meningococci strains revealed activation of caspase-3, an apoptotic route. Also, the induction of apoptosis in NG97 cells infected was demonstrated by an increased pro-inflammatory chemokines expression, such as TNF α . These data suggest that *N. meningitidis* is a useful tool in the war against cancer.

Introduction

Glioblastomas are the most common forms of primary brain tumor and the most aggressive variety of glioma [9,14,15]. Despite recent advances in the understanding of the physiopathology of this neoplasm, little progress has been achieved in treatment [6,18,22,23]. Patients with glioblastoma have poor prognosis, even when submitted to surgery, radiation and chemotherapy combined [6,22,26]. The development of successful therapeutic approaches for malignant gliomas depends on further investigations about their biology [16].

Established permanent cell lines offer an excellent opportunity to study several aspects of the glioma pathology, which may be pertinent to diagnostic and therapeutic issues [10,16]. NG97 cell line was established and partially characterized in 2001 by Grippo et al. [11,27] from a temporal grade III astrocytoma [2,5,34] obtained from a 66-year old male.

Neisseria meningitidis is the major cause of meningitis and invasive septic diseases in adult human. This Gram negative bacterium is capable of invading tissues and barriers of the upper-respiratory tract, the blood barrier and the last and more important, the blood-brain barrier. Until this date just a few studies concerning the invasion processes of these barriers by this pathogen were conducted but the major biological processes are still unclear [1,12,13]. This lack of knowledge is mainly due to the non-existence of efficient animal models capable of mimicking the human blood-brain barrier and the inefficacy to establish immortal cell lines derived from tissues of the human central nervous system. Although the meningococcal invasion and adhesion capacities had been analyzed by several authors in different cell lines such as HUVEC, Hec-1B and Hep2 cells [8,20,24,25,28,30,31].

In this work, the glioblastoma cell line NG-97 was submitted to adhesion assays using *N. meningitidis* [4], aiming on analyzing the effects of this bacterial pathogen in this astrocytoma cell line.

Material and Methods

Bacterial growth and colony-forming units (CFU) determination

The *N. meningitidis* strains used in this work (Table 1) were grown

in chocolate agar at 37°C with 5 % CO₂. For CFU determination, several colonies grown in chocolate agar were picked up with a sterile loop and re-suspended in saline solution 0.9 % (w/v) to a final optical density of 1.0. Ten-fold dilutions were performed in the same medium and aliquots of 100 μ l were plated onto chocolate agar for colonies count.

Maintenance of NG97 cell culture

NG97 cells were grown in plastic flasks (25cm²) with RPMI 1640 medium (Cultilab, Campinas, SP, Brazil), supplemented with 2 % L-glutamine, 120 μ g/mL garamycin and 13 % inactivated fetal bovine serum (complete medium). The cultures were incubated at 37°C in an atmosphere containing 5 % of CO₂. Medium was changed every 48 h and when the culture reached confluence, the subculture was performed by treatment with trypsin and versene (Adolfo Lutz, São Paulo, SP, Brazil).

Adhesion assay of *N. meningitidis* and percentual adhesion determination

N. meningitidis growth obtained in agar chocolate plates was scraped under sterile conditions, resuspended in RPMI1640 medium to an optic density of 1.00 at 600 nm and 1:100 dilutions made in the same medium. Aliquots of 50 μ l of the bacterial suspensions containing approximately 1.10⁷ CFU were submitted to the adhesion tests in the NG97 monolayer cells per well. After incubation during five hours at 37°C, the monolayers were washed five times with phosphate-buffered

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saline pH 7.2. The adherent CFU in NG97 cells were scraped and the mix plated onto chocolate agar and grown as described before. The determination of the CFU was performed in triplicate. Adhesion was calculated as the ratio of the cell-associated CFU in 50 µL of the bacterial suspension used for the infection of the cells. Adhesion assays were performed using NG97 cells as described above, after the time of infection of five hours, was performed the staining of the cells by Giemsa staining.

Scanning electron microscopy

NG97 cells were grown as described above. After the cells were fixed with 2.5 % glutaraldehyde and 4 % paraformaldehyde in phosphate buffer (pH 7.4) for 1 hour at room temperature, post-fixed in 1.0 % osmium tetroxide (OsO₄) for 10 minutes, washed in 0.1 M phosphate-buffered saline (pH 7.2) and dehydrated in a grade series of ethanol. Cover slips were critically point dried using liquid CO₂ as transition fluid. The specimens were coated with gold and observed in a JEOL JMS 5800 LV scanning electron microscope (SEM) with accelerating voltage of 10 kV.

Caspase-3 activity assay

NG97 cells were seeded in 24-well tissue culture plates and were

allowed to grow until confluence and, after that; the cells were infected in the conditions described above. The Caspase-3 activity assay followed the design described by R&D Systems (Minneapolis, MN). After 4 hours of incubation the cells were scraped and equivalent protein aliquots of each bacterial strain were determined for caspase-3 activation. All de assays were performed in triplicate. As a negative control was used NG97 cell non-infected by meningococci. Also, as positive control was used the NG97 cell cultivated at 96 hours in poorly nutrient conditions.

Production of inflammatory chemokines by Real Time PCR

For those strains that showed morphological alterations when in contact with NG97 cells, analysis of the chemokines production (IL6, IL8, IL10 and TNFα) was performed in order to verify the induced apoptosis by bacteria. NG97 cells were infected as described before e incubated for five hours. Then, the total RNA was extracted using the Trizol Reagent (Invitrogen, Calsbag, CA, USA). RNA yield was estimated by Nanodrop (Thermo Scientific). A minimum of 0.2 mg RNA was submitted to reverse transcriptase followed by detection reaction by qRT-PCR. Real-time PCR primers are listed in Table 2. qRT-PCR was performed using a StepONE Plus thermocycler (Applied Biosystems). Each 10ul reaction contained 400 nM of each primer,

Strain	Description	Adhesion percentage in NG cells	Morphologic alterations in 100 cells	Origin
C2135	<i>N.meningitidis</i> serogroup C from MERIEUX/ Instituto Oswaldo Cruz	4.70±1.24	29.67±0.58	INCQS*
P2143	<i>N.meningitidis</i> C:NT:P1.18-1,3 Cluster A4, ST8	3.03±1.02	18.33±1.33	(7)*
BATCC	<i>N.meningitidis</i> ATCC 23247 serogroup B	5.00±1.60	49.67±4.61	(7)*
W135	<i>N.meningitidis</i> ATCC 35559 serogroup W135	7.20±3.79	3.33±1.15	(7)*
P374	<i>N.meningitidis</i> serogroup Y isolated from meningitis case. Brazil, Rio de Janeiro State, Clinical isolate.	3.52±0.47	39.00±1.73	(7)*
B4	B4:P1-7,16 strain from IAL collection	58.70±6.72	67.00±2.64	IAL**
P2181	Serogroup C:NT: P1.5-1,10, Cluster A4	10.90±0.57	36.67±8.39	INCQS
P2354	Serogroup B: NT:P1.7-2,3 Cluster A4	4.48±0.05	21.67±5.69	INCQS
P2498	Serogroup C:NT:P1.5-2,10 Cluster A4, ST153	7.50±0.72	18.33±3.79	INCQS
P2200	C:NT: P1.5-2,10 Cluster A4, ST153	3.20±1.50	14.67±6.66	INCQS
Y USA	<i>N.meningitidis</i> serogroup Y strain from IAL collection	13.83±1.00	35.00±10.51	IAL
IAL 2443	B:4,7:P1-15,19, <i>N.meningitidis</i> epidemic strain from Brazilian southwest region	5.87±0.84	21.67±2.08	IAL

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** IAL – Instituto Adolfo Lutz – São Paulo, Brazil

Table 1: Strains used in this work and adhesion characteristics.

Chemokine or endogenous gene		Amplicon Size (bp)	Reference
IL6	FW GAGGATACCACTCCCAACAGACC	141	(21)
	RV AAGTGCATCATCGTTGTTTCATACA		
IL8	FW ATGACTTCCAAGCTGGCC GTGGCT	294	(21)
	RV TTATGAATTCTCAGCCCTCTTCAAAAA		
IL10	FW GTGATGCCCAAGCTGAGA	138	(21)
	RV CACGGCCTTGCTCTTGTTT		
TNF-α	FW TCTTCTCGAACCCCGAGTGA	151	(21)
	RV CCTCTGATGGCACCACCAG		
GAPDH	FW TGCACCACCAACTGCTTAGC	148	(21)
	RV GGCATGGACTGTGGTCATGAG		

FW – Oligonucleotide forward, RV- Oligonucleotide Reverse

Table 2: Oligonucleotides used in this work for qRT-PCR.

5ul Master Mix and 1/60 000 Fast EVA Green Master Mix (both from Biotium), 0,25ul of Super Script III Platinum One-Step qRT-PCR System (Invitrogen Calsbad, CA, USA), according to the recommendations for cDNA production described in the kit. Reactions were performed in triplicate and crossing threshold (ct) values were averaged. Fold change in expression was calculated according to the standard formula $2^{-(En-Rn)/(Et-Rt)}$, where En is the ct of the experimental gene in the normal sample, Rn is the ct of the reference gene in the normal sample, Et is the ct of the experimental gene in the treated sample and Rt is the ct of the reference gene in the treated sample. qRT-PCRs were repeated on three different biological replicates using as endogenous control de GAPDH gene and fold expression changes were averaged.

Statistical analysis

The data from each assay were statistically analyzed using Dunnet test compared with a control sample and $P < 0.05$ was considered significant. All experiments were performed in triplicate and the data shown in the graphs and in the table represent the means \pm standard errors.

Results

The results herein obtained performing the adhesion assays using different serogroups and lineages of *N.meningitidis* are shown in Table 1. In this way, strain B4 has demonstrated a significant adhesion to NG97 cells (glioblastoma) when compared to other meningococci strains. Also the strain C2135 demonstrated to have a higher adhesion to glioblastoma cells (NG97) when compared with strains BATCC, W135 and P374.

Optical microscopy analysis (Figure 1) of the adhesion and morphological alterations caused by C2135 infection, also detected cytoplasmatic projections called bleb's. The same effect was showed by the strains P2143; IAL 2443 and Y USA. Also these morphological modifications were quantified in each 100 cells and counted in optic microscopy. In both analyses, the B4 strain showed the major adhesion

(58%) and morphological alterations capacity in NG97 cells line (67%) (Table 1). All the strains showed adhesion in NG97 cells as well as morphological alterations.

Scanning-electron microscopy assays (Figure 3) performed with strain C2135 on NG97 cells showed that the adhesion process induced a decrease in the number of microvilli on the cell surface as well as the

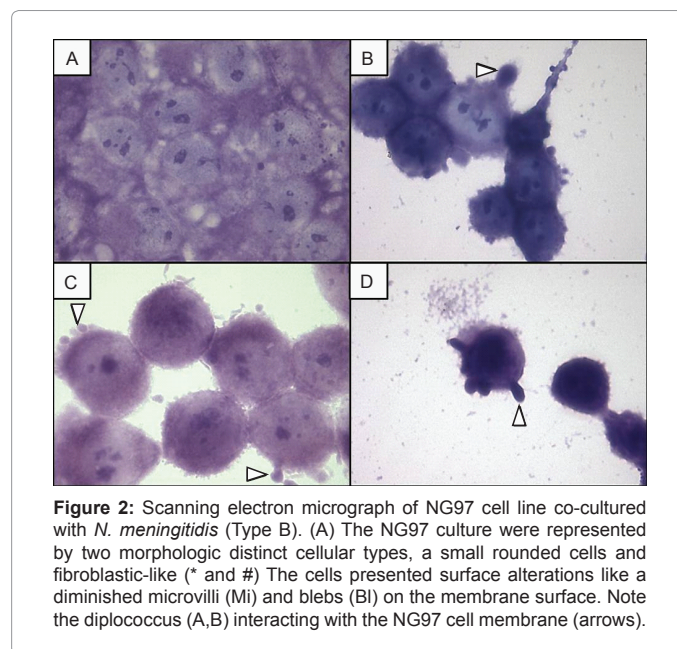


Figure 2: Scanning electron micrograph of NG97 cell line co-cultured with *N. meningitidis* (Type B). (A) The NG97 culture were represented by two morphologic distinct cellular types, a small rounded cells and fibroblastic-like (* and #) The cells presented surface alterations like a diminished microvilli (Mi) and blebs (Bl) on the membrane surface. Note the diplococcus (A,B) interacting with the NG97 cell membrane (arrows).

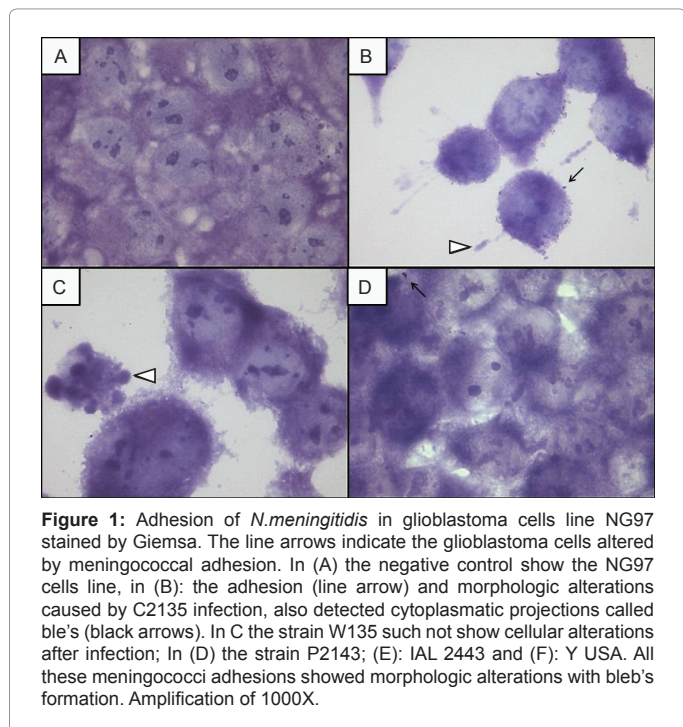


Figure 1: Adhesion of *N.meningitidis* in glioblastoma cells line NG97 stained by Giemsa. The line arrows indicate the glioblastoma cells altered by meningococcal adhesion. In (A) the negative control show the NG97 cells line, in (B): the adhesion (line arrow) and morphologic alterations caused by C2135 infection, also detected cytoplasmatic projections called bleb's (black arrows). In C the strain W135 such not show cellular alterations after infection; In (D) the strain P2143; (E): IAL 2443 and (F): Y USA. All these meningococci adhesions showed morphologic alterations with bleb's formation. Amplification of 1000X.

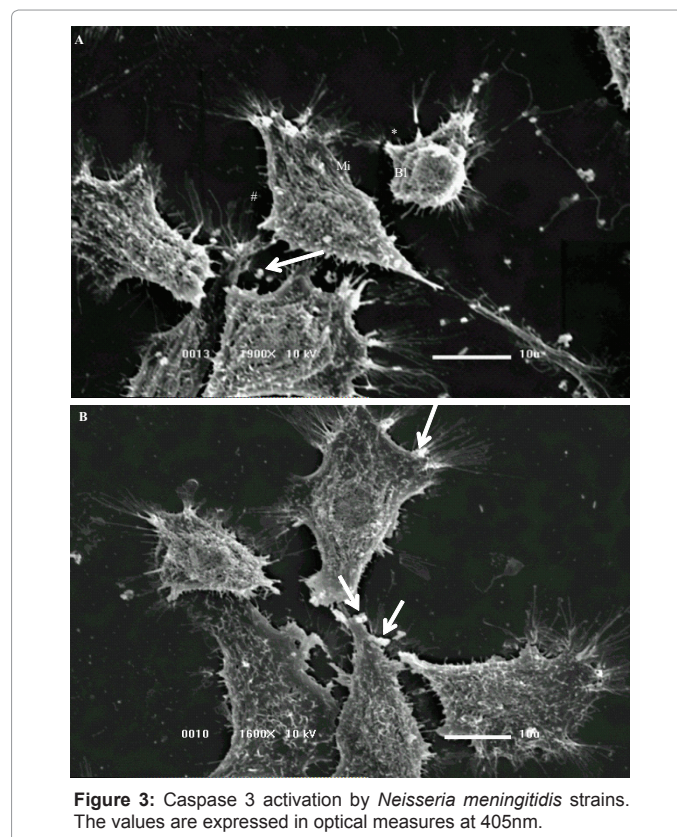
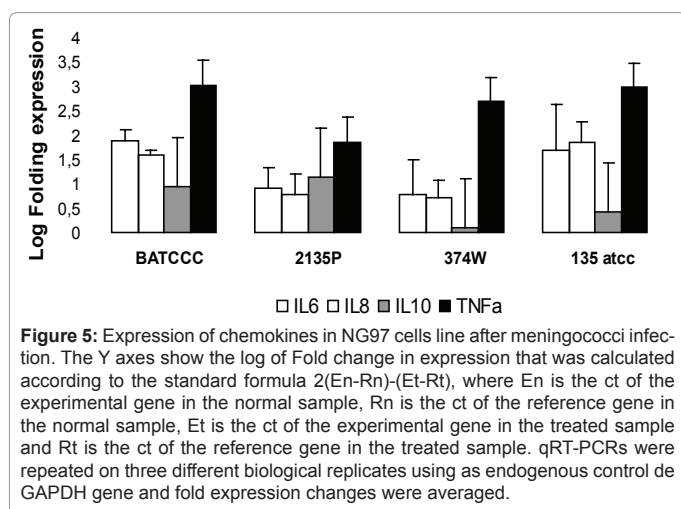
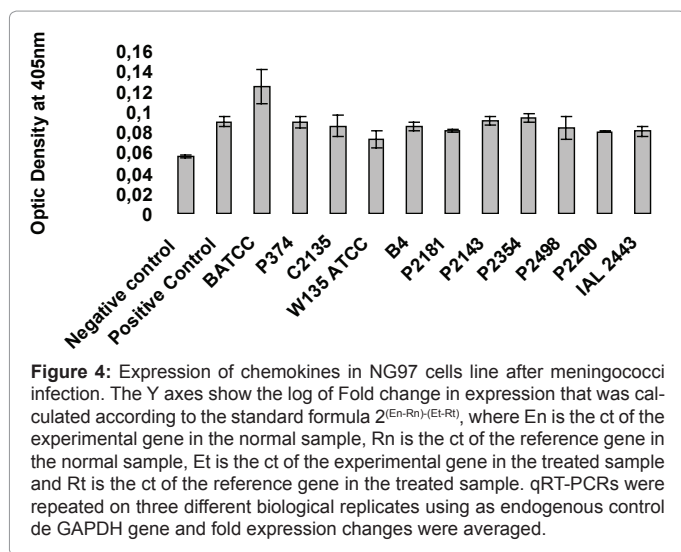


Figure 3: Caspase 3 activation by *Neisseria meningitidis* strains. The values are expressed in optical measures at 405nm.

meningococcal adhesion to the cells matrix when compared with the non-infected NG97 cells.

The analyses of the caspase-3 activation in NG97 cells infected by the different strains of *N. meningitidis* have demonstrated great activation of this apoptotic pathway in this cellular line by the strain B4 (as showed by the Figure 4). Moreover, the major activator of caspase-3 was the strain B ATCC that has also demonstrated a great adhesion and morphological alterations in these cells (Table 1 and Figure 3). Nevertheless, the least adherent strains that showed the lowest capacity to morphological alterations were the W135 strain that also showed lower caspase-3 activation.

The expression of inflammatory chemokines was performed in representative strains such as BATCC, C2135, P374 and W135 (Figure 5) due the characteristics showed in the adhesion and caspase3 activation assays. The chemokines IL8, IL6 and TNF α were detected. The anti-inflammatory cytokine IL10 expression was not detected. The expression of TNF α by NG97 cell line infected with meningococci strains B ATCC, C2135, P374 and W135 indicated the apoptotic process. The interleukines IL6, IL8 and IL10 were detected in the all strains in several folding expression as viewed in the Figure 5.



Discussion

The adhesion of *N. meningitidis* strains, important pathogens of the human central nervous and respiratory systems in glioblastoma cell line NG97, was performed to verify if these bacteria would be able to cause any pathogenic effect in these glia cells from human origin [11,27].

The TNF α increased expression in infected NG97 cell line and the caspase3 activation showed that the apoptotic process in this cells with tumor origin has been caused by meningococcal infection. All the strains analyzed share these peculiarities and induce apoptosis in these cells.

The quest of a cellular model capable to mimicry the *N. meningitidis* infectious effects under human cells have been the aim to the establishment of new meningococcal worldwide therapies. Nevertheless, the impossibility to obtain the in vivo models is an important report to the actions of meningococcal cells in human body. The search for blood-brain barrier receptor to meningococcal adhesion has been the target of several works [12,19].

There are cellular models using polarized cells as described by Virji et al. [32] but not a cellular model using cells that were derived from human glioma, as the NG97 cell line. This work showed the NG97 cells as a model to the adhesion of *N. meningitidis* considered an occasional pathogen of the nervous central system.

The adherence of this microorganism has shown not only an intimate adhesion but important alterations in the morphology of this cell line. These morphological descriptions, clearly indicate (Table 1 and Figure 1) a singular interaction between this pathogen and glia cells, it also indicates a potential of meningococcal infection and their glioma consequences in inducing the apoptosis process in these cells [17].

The interactions among several bacterial strains inducing the apoptotic process showed the efficiency [21] of this pathogen in its use as a new therapy strategy against tumor. However, some aspects of the apoptotic pathway can be elucidated, since this process was slightly detected in the NG97 cells infected by W135 strain (Figure 1,4,5). The W135 strain, in turn, showed a low pathogenicity in other cellular lines (data not shown). Hence, the great majority of meningococci tested in this work induced the apoptotic process what suggests the use of this bacterium in the war against this type of cancer, since it has been used by several authors [3,29,33,35,36].

The inflammatory chemokine expression showed that several chemokines are implicated in the inflammation of the brain cell lines in the meningitis process. Nevertheless, as shown in Figure 4, the increase of the TNF α levels in NG97 cells infected by meningococci strains, as well as the caspase-3 activation reinforced the hypothesis of the apoptotic process induction by meningococci infection in these cells. Also, the caspase-3 activation have demonstrated a quantitative analysis of the same meningococci strains as W135, for example, such strain showed the lowest caspase3 activation ($P>0.05$ considered non significant) and did not present morphological alterations in its NG97 cellular infection.

In conclusion, the cytopathological effect of the bacterial cells on the tumor opens great options for advances in the treatment or regression of these tumor cells. Also, the interactions of this bacterial pathogen with this cellular line may be an object of study to bacterial war against the tumor, specially the brain tumor.

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