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THE RELATIONSHIP OF HLA ANTIGENS TO DOXYCYCLINE INDUCED APOPTOSIS IN IMMORTALIZED B CELLS

Ping Wang [1], Gary Dorante [2], Saul R. Powell [1] and Eileen Hilton [1]

[1] Department of Medicine, Long Island Jewish Medical Center, New Hyde Park, New York 11040, [2] Department of Medicine, Weill Medical College of Cornell University, New York, New York 10021

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1. ABSTRACT

Previous studies have shown two subsets of Lyme disease (LD) patients: a seropositive group with a high frequency of the HLA class II antigen, HLA-DR7 (DR7+), and a seronegative group with a low frequency of HLA-DR7 (DR7-). The present study examined the hypothesis that the absence or presence of this antigen may play a role in the mode of B cell death induced by doxycycline. B cells, obtained from one HLA-DR7- (AL7N) and one HLA-DR7+ (MM7P) normal volunteers, were immortalized using Epstein-Barr Virus (EBV). Doxycycline resulted in a dose-dependent decrease in cell viability which was not different between the two cell lines. DNA from the MM7P showed a strong internucleosomal fragmentation pattern consistent with apoptosis, while the AL7N showed a weaker pattern, when treated with doxycycline, 20 ug/ml, for 16 hours, a result confirmed with the TUNEL assay. In the MM7P, the level of inducible p53 peaked at 8 hours while no changes were observed in the AL7N. A much higher level of HLA class II and HLA-DR was observed in the AL7N cell line which was not affected by doxycycline. These results support the conclusion that doxycycline induces p53-dependent apoptosis in MM7P. Although doxycycline induces death in AL7N, the mode and mechanism require further study.

2. INTRODUCTION

Genetic variation can predispose an individual to respond differently to infections and treatments. In a previous study, we examined HLA alleles in seronegative and seropositive Lyme disease (LD) patients, and observed that one out of 18 seronegative patients were HLA-DR7- (5.6%) and nine of 22 seropositive LD patients were HLA-DR7+ (40.9%) (1). Lyme disease is an infection caused by the tick-borne spirochete *Borrelia burgdorferi* Approximately 60% of untreated patients will develop intermittent or chronic arthritis which can usually be treated with antibiotic therapy (2). However, in about 10% of patients, arthritis will persist for months or years after antibiotic therapy (3, 4). A prior study (5) indicated that certain HLA Class II genes can determine the host immune response to *B. burgdorferi* that results in chronic arthritis and failure of antibiotic therapy.

Doxycycline is an effective antibiotic treatment for LD (6). It has been shown that doxycycline suppresses lymphocyte function (7) and immunoglobulin G (IgG) secretion (8) as well as inducing apoptosis in cultured monocytes, macrophages and osteoclasts (9, 10). Suppression of lymphocyte function could result from drug-induced cell death and in predisposed individuals may play a role in the host immune response leading to chronic arthritis and failure of drug therapy. The absence or presence of certain HLA Class II antigens can mediate B cell apoptosis (11). The present study examined the hypothesis that HLA Class II genes may modulate the vulnerability of B cells to the immuno-suppressant effects of doxycycline. Using EBV immortalized B cells from two normal controls, one possessing the HLA-DR7 (MM7P) allele and one without the HLA-DR7 (AL7N) allele, we demonstrate a differential response of these B cells to mode of doxycycline-induced cell death.

3. MATERIALS AND METHODS

3.1. Infection of primary B cell

EBV can effectively immortalize resting B cells into actively cycling lymphoblastoid cell lines (LCLs) (12). B cells were immortalized with EBV to avoid frequent blood drawing from volunteers and to obtain optimal

Table 1. MTT assay

Title	AL7N (DR7-) n = 5			MM7P (DR7+) n = 5		
Doxycycline (µg/µl)	$2^{a,x}$	20^{b}	$200^{c,x}$	$2^{a,y}$	20^{b}	$200^{c,y}$
% of control	88.1	69.4	32.1	86.7	63.6	25.1
± SD	±5.5	±8.2	±16.4	±5.9	±6.9	±14.2

SD, Standard Deviation; P>0.05 for all concentrations between cell lines; P<0.001 between 2 and 200 ug/ul doxycycline for each cell line.

conditions for consistent results. Primary B cells were purified from two normal controls from our previous study (1). Mononuclear cells were isolated by centrifuging heparinized whole blood over lymphoprep (Invitrogen, Carlsbad, CA). The mononuclear cell layers were cultured in 25 cm^2 flasks for two hours to remove monocytes and CD-19 positive B cells were selected with pan-B Dynabeads (Dynal, A.S., Oslo, Norway). After release from Detachabead (Dynal, A.S., Oslo, Norway), B cells were cultured in RPMI 1640 media supplemented with penicillin/streptomycin, L-glutamine and 10% heat inactivated fetal bovine serum (FBS) and then infected with filtered (0.45 um) supernatant from B95-8 cells (ATCC, Rockville, MD) overnight (13). The surviving cells were cultured and expanded routinely in RPMI 1640 media supplemented with penicillin/streptomycin, L-glutamine, 10% FBS and 15-20% THP-1 cell supernatant (kindly provided by Dr. Joseph Mattana, Division of Hypertension and Kidney Diseases, Long Island Jewish Medical Center). Cells were used between the 20^{th} and 40^{th} passages for our experiments.

3.2. MTT assay

Cell viability was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylthtrezolium bromide, Sigma Chemical Co., St. Louis, MO) assay as described previously (14). Cells were plated at 1×10^6/ml in replicates of four with doxycycline (American Pharmaceutical Partners, Inc, Los Angeles, CA) at concentrations of 2 ug, 20 ug and 200 ug/ml and incubated overnight. MTT was added to each well for a final concentration of 0.4 ug/µl and incubated for 2 hours at 37°C. At the end of the incubation, the medium was removed and DMSO was added to dissolve the crystals.

3.3. Determination of B cell apoptosis

B cells, 5×10^6 cells from each cell line, were incubated with 2, 20, or 200 ug/ml doxycycline in RPMI 1640 complete medium for 18 h to generate a dose response curve, after which 20 ug/ml doxycycline was selected for study of various time periods. At the end of incubation, cells were washed with PBS before being fixed in suspension with 70% ethanol and DNA fragmentation determined using the method of Gong *et al.* (15) as previously described (16). TUNEL analysis of apoptosis was performed on paraformaldehyde-fixed air-dried B cells using the *In Situ Cell Death Detection Kit* (Boehringer Mannheim Corp., Indianapolis, IN) as previously described (17).

3.4. Protein extraction and Western blot analysis

B cells, 5×10^6 cells from each line, were incubated with 20 ug/ml doxycycline in RPMI 1640 medium for 4, 8 and 16 hours then washed two times with PBS. The pellets were lysed with lysis buffer containing: Tris, 50 mM, pH ≥ 8.0; NaCl, 150 mM; 1.0% NP-40; 0.5% DOC; 0.1% SDS; PMSF, 1mM; and 1 × protease inhibitor cocktail (CalBiochem, San Diego, CA) and then centrifuged at 13,000 *g* for 5 min. Protein content of the supernatants was determined using BCA assay (Pierce, Rockford, IL). Proteins (50 ug per lane) were run on 4-20% Tris glycine PAGE gradient gels and then transferred to PVDF membranes (NEN Life Science Products, Boston, MA). Membranes were probed with antibodies specific for p53 (Oncogene Research Products, San Diego, CA), $p21^{waf1}$, $p27^{kip1}$, Bcl_2, Bax (Santa Cruz Biotechnology, Santa Cruz, CA), HLA-DR (Chemicon International, Temecula, CA) and HLA class II (Accurate Chemical and Scientific Corporation, Westbury, NY) separately. A positive control was prepared by exposing lymphocytes to 1 uM adriamycin for 4h (16). Membranes were developed using ECL chemiluminescence (Perkin Elmer Life Sciences, Boston, MA). Densitometry was performed using an IS-1000 Digital Imaging System.

4. RESULTS

4.1. Cytotoxicity

An initial evaluation of doxycycline-induced death of B cells is shown in Table 1. The data averages 5 experiments. Doxycycline treatment resulted in significant loss of viability of both cell lines, and no differences between the two cell lines were observed (Tukey-Kramer Multiple Comparisons Test).

4.2. Apoptosis

Mode of B cell death was assessed by determining the presence of internucleosomal sized fragments of DNA (DNA Ladder). In preliminary dose response experiments, DNA Ladders were detected only after treatment with doxycycline, 20 ug/ml (MM7P showed a strong ladder; AL7N showed a weaker ladder; data not shown). A time course of DNA ladder formation is presented in Figure 1 which is representative of six experiments. DNA ladder appearance was observed in MM7P cells after 8 h of treatment with 20 ug/ml doxycycline which increased after 16 h of incubation. This is in contrast to the AL7N cell line where only a smear pattern or a weak ladder pattern was observed.

These results were confirmed by Terminal transferase-dUTP-Nick-End Labeling (TUNEL). As illustrated in Figure 2, a marked difference in staining density between AL7N and MM7P was observed after a 16-hour treatment with doxycycline, 20 ug/ml. The MM7P line from the HLA-DR7+ volunteer exhibited an increase in labeling as indicated by the presence of dark-staining nuclei. This is in contrast with the AL7N line from the HLA-DR7- volunteer which demonstrated very few cells labeled. The untreated groups from both cell lines had very little stain. These results indicate that doxycycline induces apoptotic cell death in the MM7P cell line but not in the AL7N line.

4.3. Induction of apoptosis related proteins

Changes in several apoptosis related proteins were assessed using immunoblot. These proteins included

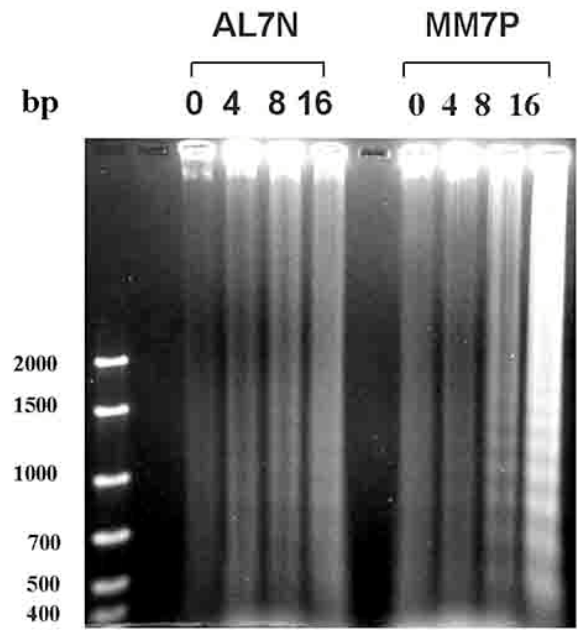

Figure 1. DNA fragmentation was observed on a 1% agarose gel. The cell lines were treated with 20 ug/ml doxycycline for 4h, 6h, and 16 h (0 was untreated). DNA ladder was detected at 16 h in MM7P cell line but in AL7N only a smear was seen.

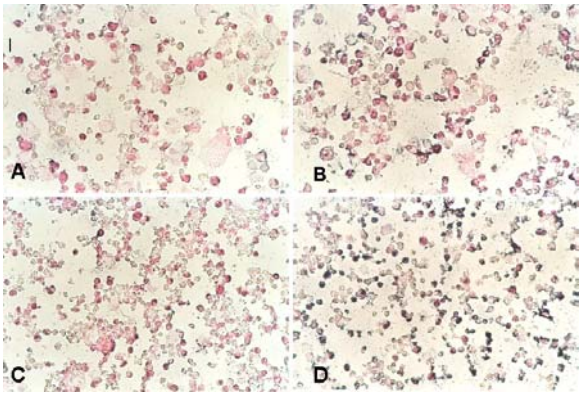

Figure 2. TUNEL staining detected apoptotic nuclei (400 ×). Untreated cells showed only occasionally positive staining nuclei: A, AL7N; C, MM7P. Cells were treated with 20 ug/ml doxycycline for 16 h showing significant numbers of positively stained cells D, MM7P and little staining B, AL7N.

the tumor suppressor protein, p53; the cyclin-dependent kinase inhibitors, $p21^{waf1}$and $p27^{kip1}$; and the cell death regulators, Bcl_2 and Bax. As illustrated by Figure 3, doxycycline treatment of the MM7P cell line resulted in a progressive increase in p53 protein that peaked at 8 hours (3.7-fold increase; one sample two-tailed t test, $P<0.05$) which was declining by 16 hours. Doxycycline treatment had no effect on p53 protein in the AL7N cell line however, these cells appear to have higher basal levels of this protein in comparison with the MM7P cell line. Doxycycline treatment had no effect on $p21^{waf1}$, $p27^{kip1}$, or Bcl_2 in either cell line. Bax did not demonstrate significant differences in either cell line as well.

4.4. Expression of HLA class II and HLA-DR

Western blot analysis revealed a much higher level of HLA class II and HLA-DR in the AL7N cell line when compared with the MM7P line (Figure 4). Doxycycline treatment had no significant effect on expression of HLA class II and HLA-DR antigens in the AL7N and MM7P cell lines.

5. DISCUSSION

Lyme disease caused by the spirochete *Borrelia burdorferi* (Bb) is a common tick borne infection in the United States and doxycycline is the most effective antibiotic used to treat this disease. Studies have demonstrated that doxycycline can induce apoptosis in monocytes, macrophages and osteoclasts (9, 10), and can suppress B cell function as well (7, 8). EBV is known for its ability to induce and maintain the proliferation of B cells both *in vitro* and *in vivo*. In fact, EBV infected B cells can maintain p53 function without disrupting it and can induce a high level of p53 and $p21^{waf1}$ without leading to growth arrest or apoptosis (13, 18). For this reason, B cells were immortalized by infecting them with EBV to carry out this study.

In this study, both DNA ladder pattern and TUNEL staining showed a marked increase in the MM7P cells at 16 hours but not AL7N cells. Interestingly, the MTT assay showed no difference in the number of viable cells between both groups of cells. These results indicate that cell death caused by doxycycline undergoes a different pathway in each of these cell lines. Our previous results provided evidence that 50% of macrophages obtained from seronegative LD patients undergo apoptosis compared to less than 10% apoptosis induction in seropositive LD patients (17). The results also showed that 41% of seropositive LD patients have HLA-DR7 and only 5.6% of seronegative LD patients have HLA-DR7 (1). Taken together, the data strongly suggest that immunogenetic profiles play an important role in the body's response to infection and treatment. Clearly, doxycycline-induced cell death in MM7P cells underwent an apoptosis pathway. The cell death pathway of the AL7N cells is not clear. It is possible that these cells undergo necrosis rather than apoptosis.

A number of genes that are involved in the apoptosis pathway have been discovered and well studied (19, 20, 21, 22). We examined p53, $p21^{waf1}$, $p27^{kip1}$, Bcl2 and Bax in this study. p53 is a tumor suppressor protein, which upregulates rapidly in response to genotoxic agents, leading to either cell cycle arrest or apoptosis (22). $p21^{waf1}$ and $p27^{kip1}$ are proteins that inhibit progression of the cell cycle by inhibition of cyclin-dependent kinases (20, 21). The Bcl2 family executes apoptosis by activating proteases called caspases. Anti and pro-apoptotic members of this family determine the life-or-death of the cell. Bcl2 protein works as a survival factor and an inhibitor of apoptosis but another Bcl2 family member, Bax, promotes cell death (19,

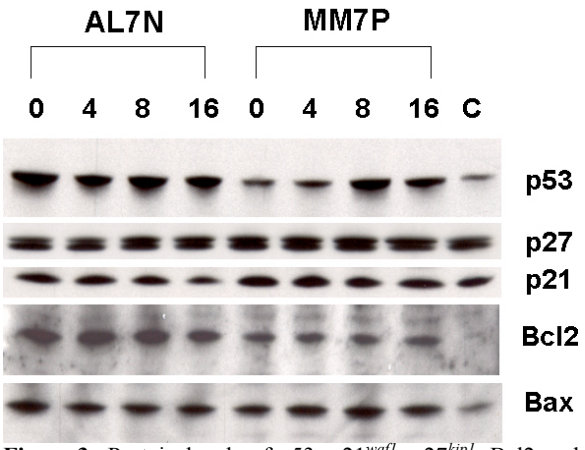

Figure 3. Protein levels of p53, p21^{waf1}, p27^{kip1}, Bcl2 and Bax were detected by Western blot. Eight hours after exposure to 20 ug/ml doxycycline, p53 protein levels increased (3.7 fold) in MM7P but not in AL7N. There were no significant changes in p21^{waf1}, p27^{kip1} , *Bc*l2 and Bax in both cell lines. C, positive control.

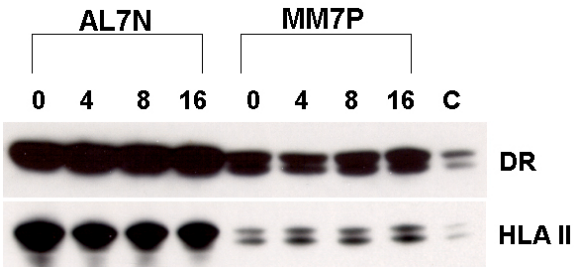

Figure 4. Protein levels of HLA class II and HLA-DR were detected by Western blot. There were no significant changes in both cell lines of HLA class II and DR but higher baseline levels of HLA class II and DR were observed in AL7N than in MM7P. C, positive control.

23). In this study, p53 protein was increased in MM7P, but p21^{waf1}, p27^{kip1}, Bcl2 and Bax were not changed. In AL7N, no significant changes in any of these apoptosis-related proteins were observed at any time point. These results suggest induction of p53 in MM7P was in response to doxycycline and acted as a mediator of apoptosis. Whether or not this modulation is controlled at the transcriptional level is the subject of future studies. The higher baseline levels of p53 in the AL7N cells may be as a result of accumulation of non-functional protein which could explain the lack of apoptosis in these cells (16, 24).

Within the HLA class II region of the DNA molecule are the genes that encode HLA-DR, -DQ and -DP. These cell surface heterodimers are constitutively expressed on human B cells (25). There is evidence for second messenger generation via the HLA class II molecule that can lead to apoptosis of B cells (11, 26). HLA-DR, but not DQ or DP, mediated apoptosis in B cells has been reported (27). In an EBV transformed B cell line, HLA-DR expression decreased when cells were treated with an oxidant (28). Our Western blots demonstrate no significant changes after doxycycline treatment in both cell lines but showed a much higher level of HLA class II and HLA-DR in AL7N than in MM7P cells. Clearly the difference between the two cell lines is due to genetic predisposition of these volunteers.

Genetic predisposition can cause an individual to respond differently to infections and treatments. Previous results demonstrated that 5.6% of seronegative LD patients had DR7 in comparison with 40.9% DR7 in seropositive LD patients (1). In the present study, doxycycline induced p53-mediated apoptosis in MM7P (DR7+) but not in AL7N (DR7-) cells which were killed by a non-apoptotic pathway that had no relation to p53 or HLA. The mechanism of cell death in this seronegative cell line is unclear but this non-responsiveness may play a role in diminished antibody production and subsequent failure of therapy in seronegative LD patients.

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Send correspondence to: Ping Wang, Lyme Disease Center, 225 Community Drive, Suite 100, Great Neck, New York 11021, Tel: 718-470-7293, Fax: 516-470-6903, E-mail: pwang@lij.edu