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Mycoplasma hyorhinis and *Mycoplasma fermentans* induce cell apoptosis and changes in gene expression profiles of 32D cells

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ABSTRACT

Infection of mycoplasmas has been linked to various human diseases including arthritis, pneumonia, infertility and cancer. While *Mycoplasma hyorhinis* and *Mycoplasma fermentans* have been detected in gastric adenocarcinomas, the mechanisms underlyine the pathogenesis are unknown. In this study, cell growth kinetics, Hoechst 33258 staining, DNA ladder assays, Western blotting analysis and cDNA microarray assays were performed to investigate the roles of *M. hyorhinis* and *M. fermentans* during infection of mammalian cells. Our data demonstrated that these mycoplasmas inhibid the growth of immortalised cell lines (32D and COS-7) ane tumor cell lines (HeLa and AGS). In addition, the infection of the 32D cell line with *M. hyorhinis* and *M. fermentans* induced compression of the nucleus, degradation of the cell genome and dysregulation of the expression of genes related to proliferation, apoptosis, tumorigenesis, signaling pathway and metabolism. Apoptosis related proteins Bcl-2, Bid and p53 were down-regulated, Fas was up-regulated and Bax was dysregulated in mycoplasma-infected 32D cells. Together, our data demonstrated that infection of mycoplasmas inhibitd cele growts through modification of gene expression profiles and post-translation modification of proliferation and apoptosis related proteins.

Key terms: mycoplasma, apoptosis, gene expression profiles.

INTRODUCTION

Microbial infection causes many human diseases, such as dermatosis, tuberculosis, and even cervical carcinoma. Previous studies have demonstrated that infection of *Helicobacter pylori* could cause gastritis and gastric cancer (Correa, 1991; Parsonnet et al., 1991; Parsonnet et al., 1994). *H. pylori* also induces apoptosis of eukaryotic cells through bacterial molecules, such as vacuolating cytotoxin A (Vac A) (Yahiro et al., 1997; Gebert et al., 2003) and CagA, which is encoded by the cytotoxin-associated gene A. These virulence factors, along with host genetic and environmental factors, constitute a complex network to regulate chronic gastric injury and inflammation, which is involved in a multistep process leading to gastric carcinogenesis (Isomoto et al., 2010; Maeda and Mentis, 2007). Other bacteria, such as the mycoplasmas that cause pneumonia, arthritis and infertility, usually exist in human respiratory, alimentary and genital tracts and they are surface epithelial parasites requiring exogenous cholesterol for membrane stability and cell entry (Metz and Kraft, 2010; Roediger, 2004; Hartmann, 2009). Mycoplasmas have also been found in patients with gastric cancer, ovary carcinoma, colon cancer, breast carcinoma and lung cancer (Huang et al., 2001; Sasaki et al., 1995), suggesting a direct link between infection of mycoplasmas and cancen. Furthermore, Feng and Tsai reported that infection of C3H (mouse embryo fibroblast) and 32D (murine myeloid cell line) cells with *Mycoplasma fermentans* induced malignancy and transformation of these cells (Feng et al., 1999; Tsai et al., 1995).

Swine are the natural hosts of *Mycoplasma hyorhinis*, which has not been previously described as a human pathogen (Decker and Barden, 1971; Magnusson et al., 1998). However, our previous work showed the presence of *M. hyorhinis* in fresh gastric tumors. Further works found that *M. hyorhinis* promotes tumor cell migration, invasion and metastasis (Yang et al., 2010).

In order to know how *M. hyorhini*e affecte host cells, we studied the growth kinetics ane gene expression profiles of mycoplasma-infected cells and found that this bacterium inhibited cell growth, induced apoptosis and dysregulated host gene expression profiles. Tiess findings will be helpful to understand further the relationship among mycoplasma infection, cell growth and cell apoptosis.

MATERIALS AND METHODS

Antibodies, proteins, interleukin 3, and other reagents

Mouse anti-β-actin monoclonal antibody was purchased from NeoMarkers (Fremont, CA, USA). Goat anti-p53 polyclonal antibody, mouse anti-Bcl-2 monoclonal antibody, ant-Bax, anti-Bid and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA), Trizol reagent from Invitrogen (Carlsbad, CA, USA), [α-^{32}P]dCTP (deoxycytidine triphosphate) from Furui Co. (Beijing, China), recombinant murine interleukin 3 (IL-3) from PeproTech EC Ltd (London, UK), Hoechst 33258 from Sigma-Aldrich (St. Louis, MO, USA) and RNase A from Zhongshan Biotechnology Company (Beijing, China).

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Mycoplasma strains and culture medium

M. fermentans (incognitus strain) and *M. hyorhinis* (ATCC 17981) came from Dr. Zhanggai Guo (Capital Children Institute, Beijing, China). The mycoplasmas were grown in modified G-PPLO medium (67.2% PPLO, 20% rabbit serum, 10% yeast extract, 1% glucose, 0.2% phenol red, 1.6% NaOH, and 1,000 U of penicillin/ml).

Cells, cell culture, and cell infection with mycoplasmas

32D cells (murine hematopoietic cells) and WEHI-3B cells were provided by Dr. Zhang (Armed Forces Institute of Pathology, Washington, D.C. USA). The former were maintained in modified RPMI 1640 medium supplemented with 15% fetal calf serum and 5% WEHI-3B conditioned medium, which contained IL-3. WEHI-3B cells were maintained in RPMI 1640 medium supplemented with 15% fetal calf serum. Mycoplasmas were resuscitated from -80°C storage, grown in modified G-PPLO medium, passed twice, and then applied to infect cells in a ratio of 1,000 color change units/cell. COS-7 and HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. AGS cells were grown in F12 nutrient medium with 10% fetal calf serum.

Analysis of cell apoptosis

(1). Hoechst 33258 staining. Mycoplasma free 32D cells, *M. fermentans*-infected and *M. hyorhinis*-infected 32D cells were harvested by centrifuge, washed by serum free RPMI 1640 once, suspended with 500 μl 2 μg/ml Hoechst 33258 in serum free RPMI 1640 for 10-15 minutes at 37°C. Then stained 32D cells were checked with a fluorescence microscope. (2). DNA ladder analysis. 32D cells were infected with mycoplasmas for 10 weeks, harvested by centrifuge and lysed in RIPA buffer (25 mM Tris•HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Total DNAs were extracted with phenol/chloroform, digested with RNase A for 1 hour at 37°C to remove RNAs and analyzed by electrophoresis on 1.8% agarose gel. (3). Western blot. Mycoplasma free and mycoplasma-infected 32D cells were harvested by centrifuge, washed with phosphate-buffered saline (PBS) and lysed with RIPA buffer. 50 μg total proteins were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with antibodies against cell apoptosis related proteins and developed with enhanced chemiluminesence (ECL) system (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA).

cDNA microarray and clustering analysis

More than 1×10^7 mycoplasma free 32D cells, *M. fermentans*-infected and *M. hyorhinis*-infected 32D cells were collected, respectively, and total RNAs were extracted with the Trizol reagent according to the manufacturer's protocol. cDNA microarrays were manipulated by using 8 BiostarM-80s chips containing 8,000 sequenced mouse cDNAs (Biostar Genechip Inc, Shanghai, China). Images were collected using a scanner (ScanArray 4000; Packard BioScience, Billerica, MA, USA) loaded with GenePix Pro 3.0 software (Molecular Devices Corporation, Union City, CA, USA). To control for dye (Cy3 and Cy5)-dependent biases within this system, two of the hybridizations were repeated with the fluorescent labels reversed. For all experiments, gene expression was measured by hybridization of fluorescently labeled samples to cDNA microarrays. Up-regulated and down-regulated genes were determined using the significance analysis of microarray algorithm (SamCluster software; available at wujuli@yahoo.com).

RT-PCR analysis

More than 1×10^8 each of mycoplasma free 32D cells, *M. fermentans*-infected and *M. hyorhinis*-infected 32D cells were collected, and total RNAs were extracted using the Trizol reagent. First-strand cDNAs were synthesized with 15 μg of total RNAs, 0.5 μg of random primers (Promega Corporation, Madison, WI, USA), 3 μl of ribonuclease inhibitors (Takara Biotechnology Co. Ltd, Dalian, China), 1 μg of oligo (dT) (Takara Biotechnology Co. Ltd), and 5 U of MMLV RT (Invitrogen) in a 50 μl RT (reverse transcription) reaction mixture according to the manufacturer's instructions. PCR amplifications were performed in a 25 μl reaction mixture for 30 cycles with the cDNA from the RT as template and the special primers (listed in Table I) and at different annealing temperatures, depending on the genes involved. PCR products were stored at -20°C for later use.

Northern blot analysis

Total RNAs were extracted from different 32D cells with the Trizol reagent as described above, and were quantified by spectrophotometry. 15 μg of total RNAs was denatured, size-fractionated by electrophoresis on a formaldehyde agarose gel, and transferred onto positively charged membranes (Hybond-N^+; Amersham Biosciences, Buckinghamshire, England). The RNA was cross-linked to the membrane with an ultraviolet crosslinker (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA) and heateg at 80°C for 2 hours. The PCR products were purified by using a DNA gel extraction kit (Qiagen, Hilden, Germany), labeled with [α-^{32}P] deoxycytidine triphosphate by random priming with Prime-a-Gene Labeling System (Promega) and purified with a PCR purification kit (Qiagen, Hilden, Germany). Blots were hybridized with labeled cDNA probes in ExpressHyb Hybridization Solution (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Membranes were then washed in 1× standard saline citrate (4.41 g Tris-sodium citrate, 8.77 g NaCl, 1,000 ml H_2O, pH 7-8) with 0.5% (w/v) sodium dodecyl sulfate and exposed to autoradiography for one day to two weeks. As a control to demonstrate RNA integrity, membranes were rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase cDNA probe. The blots were washed in sterile water containing 0.5% sodium dodecyl sulfate at 90-100°C for 15 minutes and then exposed to x-ray film to confirm the removal of the probe before the blots were reused. Density values were normalized against signals for 28S and 18S rRNA.

Statistical analysis

The data were analyzed by Pearson's c^2 test or nonparametric tests using commercially available software (SPSS 10.0). Significance was defined as $P<0.05$. Data are presented as mean ± standard deviation (SD).

TABLE I
Forward and reverse primers for genes identified by reverse-transcriptase polymerase chain reaction and Northern blot analysis

GenBank accession no.	Annotated genes	Forward Primer	Reverse Primer
AJ 131777	Src-like adaptor protein	TGATGGTCTATGCTGTGTG	CTCTGATCAGTGTGTAAAG
NM_018882	G protein-coupled receptor 56	TGAAGAACAACTCAGACAG	CAGCTCCTAAGGTGCTCTG
NM_007792	Cysteine-rich protein 2	TTCCTGTGCATGGTTTGCAG	ATTCTCTGTCGTGCTTACTG
NM_013842	X-box binding protein 1	AGACTGCTCGAGATAGAAAG	TGGTCAAAACGAATGAGTTC
NM_008363	Interleukin 1 receptor-associated kinase	AAGAAGCACCTGGACTCCAG	AGCTAGCCTCTCTGAAGGAC
NM_013492	Clusterin	AAGTTCTATGCACGTGTCTG	CATCTTCACCTTCTCTTAAG
NM_008458	Kallikrein binding protein	TGCTGGTGAATTACATCTAC	AGACTGGTTCTTCTACTAAG
NM_022332	Suppression of tumorigenicity 7	TCGGTACACTTGGGTCACAG	CTGTGTTGAGCTTCATACTG
NM_009895	Cytokine inducible SH2-containing protein	ATCTTGTCCTTTGCTGGCTG	AAGCTAGAATCGGCGTACTC
NM_010330	Embigin	TACAAGTCTACCTGTTCGAG	CACGTAAGCGATCAACGAC
NM_009061	Regulator of G-protein signaling 2	GTTTGAGCTACTTCTTGCAG	CTGACTTCCTGATTCACTAC
NM_013470	Annexin A3	TCATGGTGGCTCTTGTTAC	TGCTGTCCTCAATGTCCTTC
BC 013310	Glyceraldehyde-3-phosphate dehydrogenase	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

RESULTS

Mycoplasmas inhibit growth of cell lines

32D cells are murine hematopoietic cells and have been used to study spontaneous transformation after infection of *M. fermentans* (Feng et al., 1999), In order to determine whether infection of *M. hyorhinis* could induce apoptosis or malignant transformation of mammalian cells, we used mycoplasma free and mycoplasma-infected immortal (32D and COS-7) or cancer cell lines (HeLa and AGS) for proliferation assays. Our data showed that infection of 32D, COS-7, HeLa and AGS cells with *M. fermentans* and *M. hyorhinis* inhibitee growth rates based on comparisong to control cells (free of mycoplasmas) (Figure 1). The inhibitory effect oy *M. hyorhinis* on the growth rates of 32D and AGS cells was stronger than that of *M. fermentans* (Figure 1).

Mycoplasma infection induces apoptosis of 32D cells

When 32D cells were infected with *M. fermentans* and *M. hyorhinis* and cultured for more than 3 days, the morphology of mycoplasma-infected 32D cells changed. Most of them became smaller than that of pathogen-free cells, indicating cell shrinkage. As shown in Figure 2A, Hoechst 33258 staining showed that there were many small brilliant-blue dots dispersed in the nucleus of mycoplasma-free 32D cells; for *M. fermentans*-infected cells, the whole body of nucleus became brighter, denser and smaller, and the brilliant-blue dots almost disappeared; for *M. hyorhinis*-infected cells, there were much brighter large dots in the nucleus and the nuclei of some *M. hyorhinis*-infected cells became much larger, indicating enhanced DNA breakage in the nuclei that were associated with the shrinking of cell bodies. In some nuclei of *M. hyorhinis*-infected 32D cells, the nucleic substance moved to one side and became denser, whict meant apoptotic corpuscles, and some nuclei, were broken. This indicates that there were much more apoptotic cells in culture of *M. hyorhinis*-infected 32D cells. Even after 32D cell culture conditions were changed, such as the type of serum was changed, or the culture volume was increased, thereewere some apoptotic cells in mycoplasma-infected 32D culture.

Correlated with Hoechst 33258 staining for apoptosis, our data showed stronger DNA fragmentation in 32D cells infected with *M. hyorhinis* compareg to infection with *M. fermentans*, which was indicated by a clear DNA ladder in *M. hyorhinis* infected cells but no or a very weak DNA ladder in *M. fermentans* infected cells (Figure 2B).

Next, we analyzed the expression of several proteins related to apoptosis. In *M. fermentans* infected 32D cells, anti-apoptotic Bcl-2 was down-regulated apparently, but the expression of pro-apoptotic Bax appeared to be up-regulated. These data indicate that the *M. fermentans* infected cells were in an early stage of apoptosis. The expression of Bcl-2 in *M. hyorhinis* infected cells was also down-regulated apparently, but both pro-apoptotic proteins like Bax and Bid were down-regulated to a much greater extent, indicating a much stronger apoptosis induction by *M. hyorhinis*. This suggests that *M. hyorhinis* infected cells were in a late stage of apoptosis. We found that Bid was truncated at the early stage of apoptosis and degraded in cells at the late stage. The expression of p53 was also down-regulated in apoptotic cells. We did not detect FasL expression, but found slightly up-regulated Fas protein in mycoplasma-infected 32D cells (Figure 2C). The intensity of each protein band detected by Western blot was calculated with Photoshop CS 9.0 software and data are shown in Table II.

Change of gene expression profiles in mycoplasma-infected cells

In order to identify the genes responding to mycoplasma-infection, 32D cells, infected with *M. hyorhinis* or *M. fermentans* for four weeks and nine weeks, were used for cDNA microarray assays, respectively (Table III). The result from experimental replicates demonstrated a high degree

of reproducibility (correlation coefficient 0.872-1.033) and the profiles of the gene expression were clearly different among cells infected for various time lengths (Supplementary Materials_Excel 1-8). For First and Second chips, there were 153 differentially expressed genes with a ratio 2 or 0.5 and the same trend of up-regulation or down-regulation. For Third and Fourth chips, there were 269 differentially expressed genes, for Fifth and Sixth chips, 245, and for Seventh and Eighth chips, 98 (Linked Results in Supplementary Materials).

According to the report of Feng (1999), after 4 to 5 weeks, 32D cellseare malignantly transformed by *M. fermentans*, indicating that thehperiod of 4 weeks is the turning point of malignant transformation of 32D cells, so we chose 32D cells infected with *M. fermentans* or *M. hyorhinis* for 4 weeks and 9 weeks to extract total RNAs for cDNA microarray assays. At 4 weeks, infection of mycoplasma enhanced protein degradation and apoptosis of 32D cells by down-regulation of Serine (or cysteine) proteinase inhibitor, F-box and WD-40 domain protein and Serine protease inhibitor 1-3, and up-regulation of Proteosome 26S subunit (non-ATPase), Proteosome subunit alpha5, Ubiquitin-conjugating enzyme 8, Cathepsin and Kallikrein binding protein. Septin 6 and Inhibitor of DNA binding 4 were up-regulated because of DNA damage in 32D cells and up-regulation of Complement component 3 and Fc receptor might contribute to inflammation. Down-regulation of Src-like adaptor protein, Regulator of G-protein

TABLE II
Intensity of protein bands detected by Western blots

Proteins	Mean intensity±SD		
	Ctr-32D	Mf-32D	Mhy-32D
Bcl-2	238.87±27.17	99.03±32.34	69.41±36.69
Bax	29.65±12.40	98.86±36.48	19.04±6.59
p53	182.14±39.96	149.25±40.0	141.34±33.32
Bid	230.19±39.78	169.5±29.82	17.64±9.29
Fas	145.4±36.01	165.64±38.55	181.18±41.86
β-actin	243.11±29.48	246.04±21.38	234.96±38.91

Abbreviation: Ctr-32D, mycoplasma-free 32D cells used as control cells; Mf-32D, *M. fermentans*-infected 32D cells; Mhy-32D, *M. hyorhinis*-infected 32D cells.

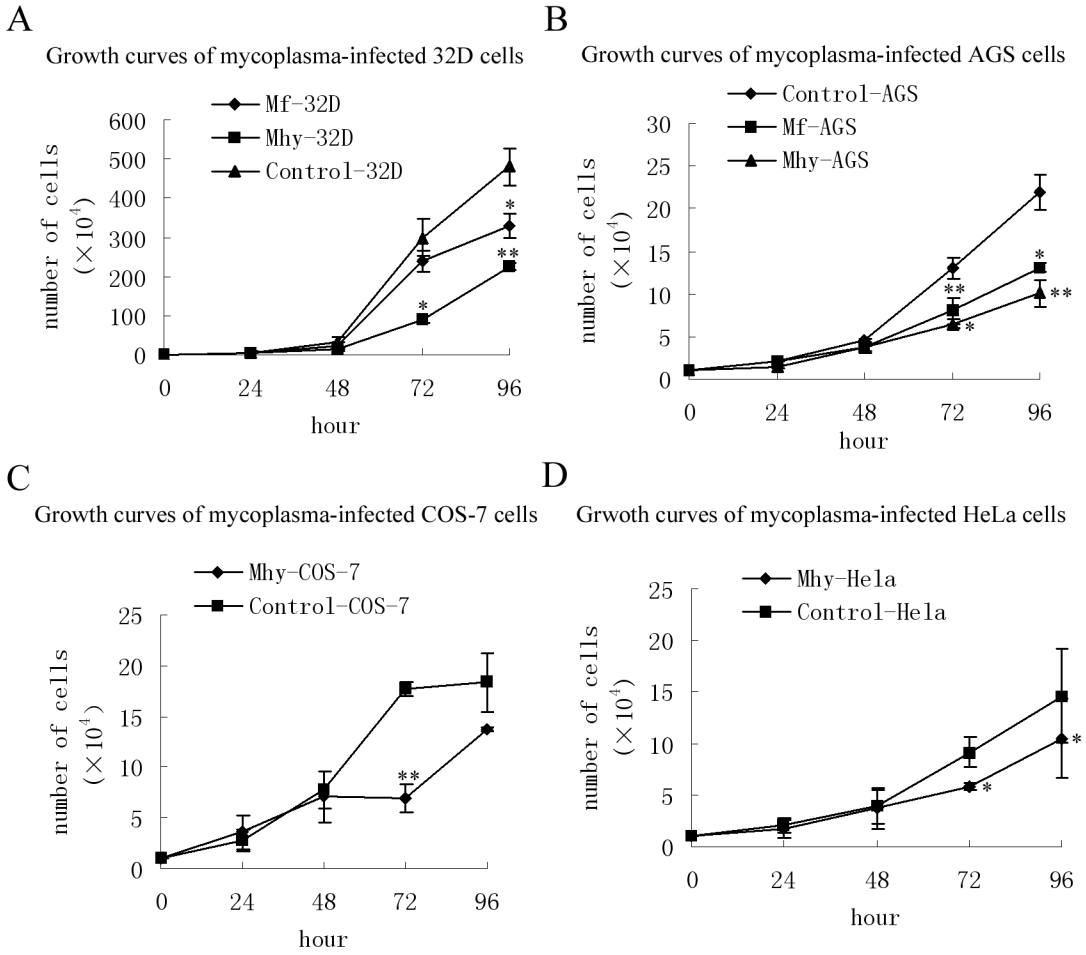

Figure 1: Growth curves for mycoplasma free or infected cells. *Mycoplasma hyorhinis* (Mhy) or *Mycoplasma fermentans* (Mf) infected 32D (**A**), AGS (**B**), COS-7 (**C**) and HeLa (**D**) cells. Control cells mean mycoplasma free cells. The assays were performed in triplicate and experiments were repeated on two separate occasions. Error bars represent SD. An asterisk (*) indicates a significant difference at $P<0.05$, compared to control cells. Double asterisks (**) indicate $P<0.01$, compared to control cells.

TABLE III
Sample arrangement for cDNA microarray chips

Chip No.	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth
Samples	4w Mf/Ctr	4w Mf/Ctr	4w Mhy/Ctr	4w Mhy/Ctr	9w Mf/Ctr	9w Mf/Ctr	9w Mhy/Ctr	9w Mhy/Ctr

Abbreviation: 4w, mycoplasma infected for 4 weeks; 9w, mycoplasma infected for 9 weeks; Mf, *M. fermentans*-infected 32D cells; Mhy, *M. hyorhinis*-infected 32D cells; Ctr, mycoplasma-free 32D cells.

signaling 2, G protein-coupled receptor 56, Suppression of tumorigenicity 7, TGF beta inducible early growth response, Cysteine-rich protein 2, Neuroblastoma myc-related oncogene 1, and up-regulation of Teratocarcinoma-derived growth factor, Cytokine inducible SH2-containing protein and X-box binding protein 1 might contribute to cell malignant transformation. At 9 weeks (after malignant transformation of 32D cells), infection of mycoplasma balanced protein degradation and cell apoptosis by down-regulation of Proteosome subunit beta 9, F-box and WD-40 domain protein 4, Ribosomal protein L22, Serine protease inhibitor 1-3, Eukaryotic translation initiation factor 2 alpha kinase, Ubiquitin-protein ligase UBE3B, and up-regulation of Cytochrome C, Caspase 7, Eukaryotic translation elongation factor 1 epsilon 1, Cathepsin, Ribosomal protein (large P2), Kallikrein binding protein and Serine (or cysteine) proteinase inhibitor. The regulation of DNA damage, inflammation and tumorigenicity related genes were similar to that of time point of 4 weeks (Supplementary Materials_Excel 1-8).

We performed gene clustering using significance analysis based on the microarray algorithm SamCluster program. Genes with similar expression patterns were clustered together. The different experiments were also clustered, and a dendrogram, illustrating the relationship between the expression patterns, is shown with arrays that have the most similar gene expression profiles and are clustered together with the shortest branches. As welld more than 200 genes were found that responded to mycoplasma infection significantly (Figure 3).

Because we found that mycoplasmal infection inhibited growth of mammalian cells and induced apoptosis of 32D cells, we examined some important genes related to cell proliferation and apoptosis. Twelve genes were selected for further investigation (Table I). Killikrein binding protein is an EGF binding protein and inhibits serine proteinase (Gao et al., 2003). It was up-regulated dramatically in all *M. fermentans* or *M. hyorhinis*-infected 32D cells. Regulator of G-protein signaling 2, which inhibits signal transduction by increasing the GTPase activity of G protein alpha subunits (Chen et al., 1997; Reif and Cyster, 2000) was down-regulated in all *M. fermentans* or *M. hyorhinis*-infected 32D cells. Src-like adaptor protein is related to the Eck receptor tyrosine kinase, has SH3 and SH2 domains, and controls mitogenesis, T cell receptor signaling and Src protein function negatively (Holland et al., 2001; Lebigot et al., 2003). Cysteine-rich protein 2 is a ligand of the ERBB tyrosine kinase receptor and has an EGF (epidermal growth factor)-like domain. It interacts with TGFB1I1 (Kim-Kaneyama et al., 2005). G protein-coupled receptor 56 is involved in the development of gastric cancer (Okumura et al., 2004). Suppression of tumorigenicity 7 may act as a tumor suppressor (Hooi et al., 2006). They were all down-regulated in *M. fermentans* (9 weeks) or *M. hyorhinis* (4 and 9 weeks)-infected 32D cells. IL-1 receptor-associated kinase is involved in IL-1 biochemistry (Qin et al., 2004). Annexin A3 is an inhibitor of phospholipase A2, possesses anti-coagulant properties, and binds calcium and phospholipids (Sopkova et al., 2002). X-box binding protein 1 is a transcription factor and is involved in the differentiation of plasma cells, in

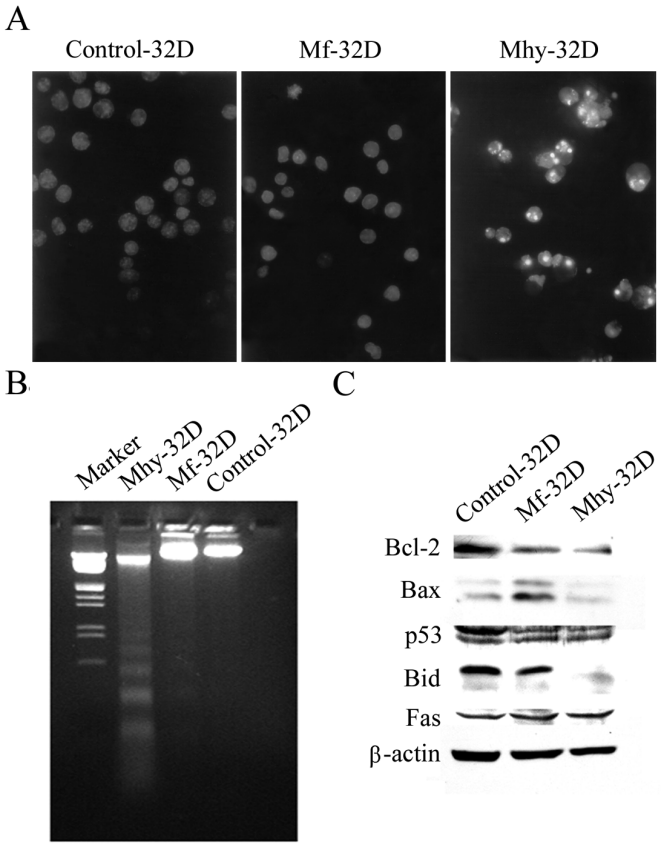

Figure 2: Apoptosis analysis of mycoplasma-infected 32D cells. Hoechst 33258 staining: Mycoplasma free 32D cells (Control-32D), *M. fermentas*-infected 32D cells (Mf-32D) and *M. hyorhinis*-infected 32D cells (Mhy-32D) were harvested and stained with 2 μg/ml Hoechst 33258 in serum free RPMI 1640 for 10-15 minutes at 37 (**A**). DNA ladder analysis: The full length DNAs were extracted from mycoplasmas infected and control 32D cells and separated on a 1.8% agarose gel. λ *Hin*d III/*Eco*R I DNA ladder was used as DNA size marker (**B**). Protein analysis. 50 μg total proteins were extracted from mycoplasmas infected and control 32D cells, separated by SDS-PAGE and detected by enhanced chemiluminescence. Western blots were performed to detect the expression of genes related to apoptosis. β-actin was used as an internal reference (**C**). The assays were performed in three separate experiments.

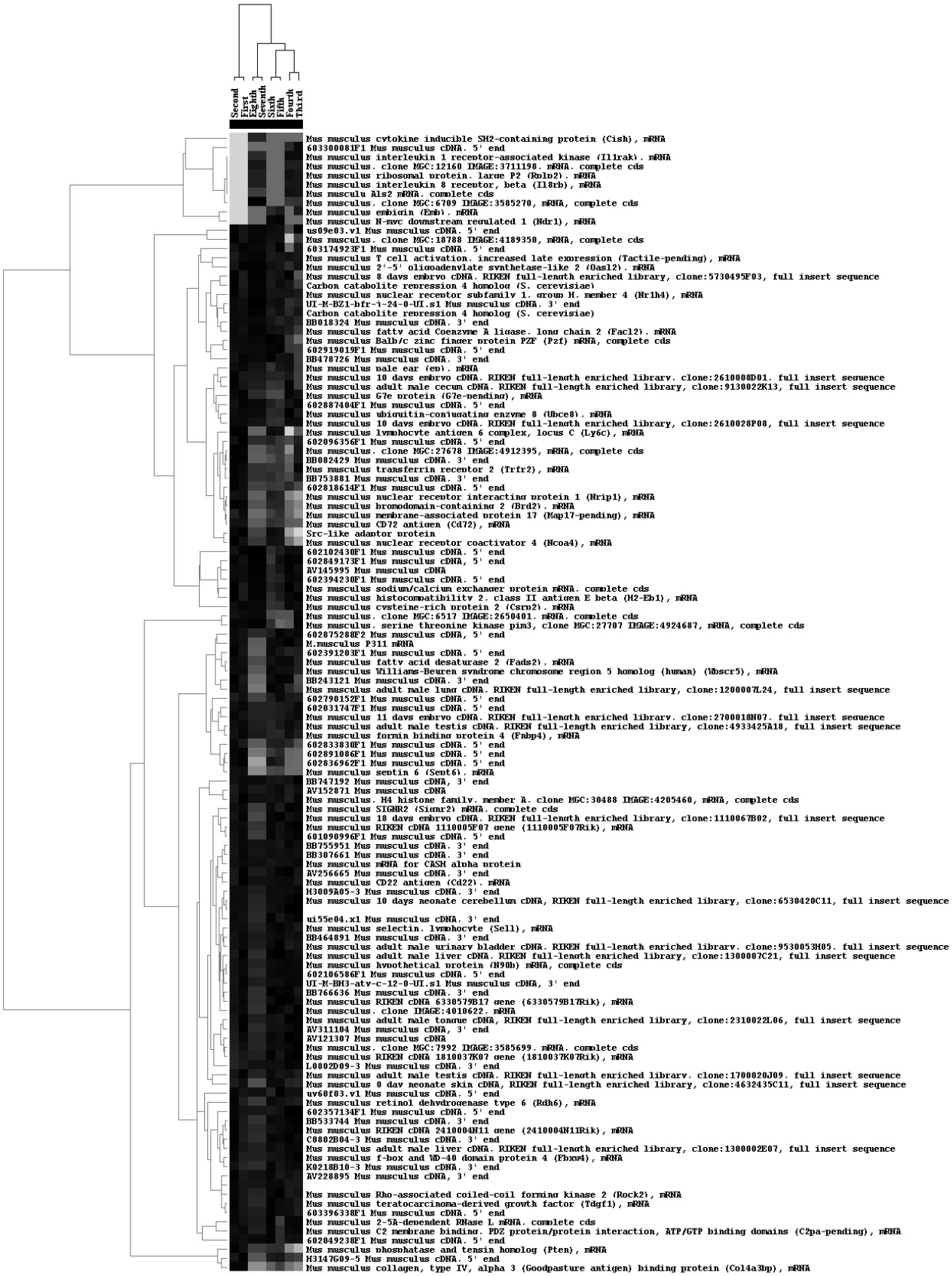

Figure 3: Hierarchical clustering analysis of gene expression patterns based on expression data. Normalized expression values for the microarrays were clustered with microarray algorithm SamCluster program. Vertical lines refer to cDNA microarray chips numbered First, Second, Third, Fourth, Fifth, Sixth, Seventh and Eighth, and each horizontal line refers to a gene. The colors represent the logarithmic intensity of the expressed genes. Red and green colors denote up- and down-regulated genes, respectively.

endoplasmic reticulum stress, and in carcinogenesis, and is a component of the BMP signaling pathway (Fujimoto et al., 2003; Takahashi et al., 2002). Embigin may function as a regulator of cell/ECM interactions during development and in the homeostasis of normal adult tissues. Cytokine inducible SH2-containing protein forms part of a classical negative feedback system that regulates cytokine signal transduction. And it is involved in the negative regulation of cytokines that signal through inhibition of STAT5 trans-activation by suppressing its tyrosine phosphorylation (Matsumoto et al., 1997). They were all up-regulated in *M. fermentans* (9 weeks) or *M. hyorhinis* (4 and 9 weeks)-infected 32D cells. Clusterin expression distinguishes follicular dendritic cell tumors from other dendritic cell neoplasms. Clusterin/apolipoprotein J (CLU) is a secreted heterodimeric glycoprotein that is up-regulated during tumorigenesis, as well as during cell injury or death (Grogg et al., 2004; He et al., 2004; Trougakos and Gonos, 2004).iIt was only up-regulated in 32D cells after long-tere (9 weeks) infection of mycoplasmas.Results of above genes from cDNA microarrays, which indicated gene expression's dysregulation, were also confirmed with Northern blots (Figure 4) and the densitometric analysis of the bands (Table IV).

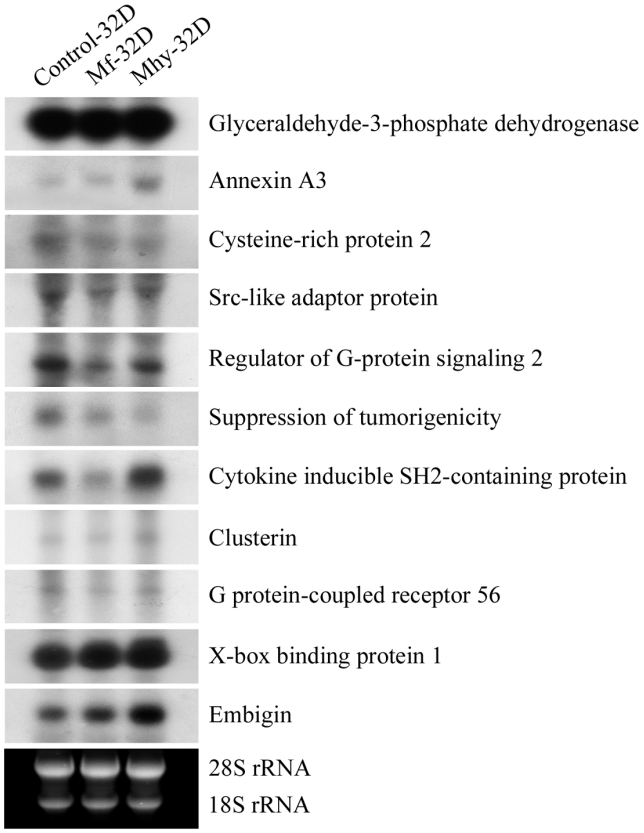

Figure 4: Identification of mycoplasma-infection-specific genes related to proliferation, apoptosis and tumorigenesis with Northern blots. Total RNAs were extracted from mycoplasma-free 32D cells (Control-32D), *Mycoplasma fermentans*-infected 32D cells (Mf-32D) and *Mycoplasma hyorhinis*-infected 32D cells (Mhy-32D). GAPDH, 28S rRNA and 18S rRNA were used as internal RNA loading controls. The assays were performed in duplicate and experiments were repeated twice.

DISCUSSION

Previous studies have demonstrated that infection of *H. pylori* induces cell apoptosis by Vac A protein (de Freitas et al., 2004; Galgani et al., 2004; Menaker et al., 2004; Cho et al., 2003; Fischer et al., 2004) and Cag A (Isomoto et al., 2010; Maeda and Mentis, 2007). Our previous studies showed the presence of *M. hyorhinis* and *M. fermentans* in fresh gastric cancer samples, but whether the infection of these mycoplasmas could directly induce gastric cells apoptosis, ulcer or cancer is unknown.

In their research, Feng and colleagues (1999) did not study if *M. fermentans* inhibits growth or induce apoptosis before the malignant transformation of 32D cells. In our research, we used *M. fermentans* as a control bacterium and used 32D cell as a cell model. We followed the protocols described by Feng (1999) and explored if *M. hyorhinis* could inhibit growth, induce apoptosis or malignant transformation of mammalian cells. Yang (2010) has reported that infection of mammalian cells with *M. hyorhinis* could promote cell migration, invasion and metastasis. Consequently, our current experiments focus on cell apoptosis caused by *M. hyorhinis*. We found that both *M. fermentans* and *M. hyorhinis* induced apoptosis of 32D cells and dysregulated gene expression profiles in 32D cells. Especially *M. hyorhinis* inhibited growth of mammalian cells much more than *M. fermentans* and these results were consistent with our apoptosis analysis. When infected the mammalian cells with mycoplasmas for less than 48 hours, the growth of cells free of mycoplasam was similar to that of mycoplasma-infected cells. That means the transcription of some apoptosis genes, translation and post-translation modification of some apoptosis proteins are just starting. After 72 hours, we observed apparent growth inhibition of mycoplasma-infected cells. That means some of cells have undergone apoptosis. These results correlate with cDNA microarray assays, indicating that infection of mycoplasmas induce dysregulation of some proliferation or apoptosis-related genes that lead to decreased cell growth. Using Hoechst 33258 staining, DNA ladder assay and protein expression analysis, we have shown that some mycoplasma-infected 32D cells underwent apoptosis as shown by the data from Bax and Bid protein analysis. In Northern blot analysis, the band of cytokine inducible SH2-containing protein (from mRNA of *M. fermentans*-infected 32D cells) is not consistent with the data of the cDNA microarray. It is possibly because of manipulation error from the cDNA microarray or from Northern blot. The bands of the other nine genes are consistent with the data from the cDNA microarray with a validation ratio of 95%. From the data of cDNA microarray, we know that mycoplasma infection induced expression of protein degradation or cell apoptosis related genes. In *M fermentans* or *M. hyorhinis*-infected 32D cells, some protease inhibitors (e.g. serine or cysteine protease inhibitor) were down-regulated. But thiol protease Cathepsin S was just up-regulated only in *M. hyorhinis*-infected 32D cells.

Infection of mycoplasmas can simultaneously induce two contrary outcomes in host mammalian cells simultaneously: cell malignancy and immortalization, or cell apoptosis. It is possible that infection of mycoplasmas will kill the weakest host cells, and transform only small percentages into malignant tumor cells, such as cancer stem-like cells. Similarly, when treated with carcinogen phorbol ester for four weeks, the growth of 32D cells was inhibited (data not shown). Together, these data suggest that infection of mycoplasma or treatment

TABLE IV
Intensity of mRNA bands detected by Northern blots

GenBank accession no.	Annotated genes	Mean intensity±SD		
		Ctr-32D	Mf-32D	Mhy-32D
BC 013310	Glyceraldehyde-3-phosphate dehydrogenase	245.58±4.03	246.74±3.25	246.15±3.34
NM_013470	Annexin A3	68.11±7.44	69.4±8.78	96.44±11.9
NM_007792	Cysteine-rich protein 2	143.1±15.7	118.69±9.84	111.59±11.62
AJ 131777	Src-like adaptor protein	167.58±26.26	131.02±19.04	149.81±24.12
NM_009061	Regulator of G-protein signaling 2	173.22±38.95	132.39±26.85	137.44±37.23
NM_022332	Suppression of tumorigenicity 7	117.52±20.87	91.63±13.82	82.31±8.44
NM_009895	Cytokine inducible SH2-containing protein	113.84±34.79	90.16±16.75	155.58±35.91
NM_013492	Clusterin	56.51±10.45	62.34±4.94	83.71±14.06
NM_018882	G protein-coupled receptor 56	106.52±18.1	81.73±6.96	89.29±15.69
NM_013842	X-box binding protein 1	189.61±30.21	216.2±18.76	227.37±11.87
NM_010330	Embigin	134.34±35.83	178.31±29.24	221.71±25.64

Abbreviation: Ctr-32D, mycoplasma-free 32D cells used as control cells; Mf-32D, *M. fermentans*-infected 32D cells; Mhy-32D, *M. hyorhinis*-infected 32D cells.

with mutagen induces host cell apoptosis prior to the induction of transformation and malignancy. Other studies have shown that infection of mycoplasmas can promote carcinogenesis by other pathways, such as promoting metastasis (Yang et al., 2010). Mycoplasma can also control the size of tumors by unknown mechanisms. Thus, by inducing transformation of host cells into tumor cells, mycoplasma can evade the immune surveillance by modification or dysregulation of host cell proteins that cannot be recognized by the host immune system. Further works are needed to characterize the relationship among mycoplasma, gastric cancer and other gastric diseases, such as gastric ulcers and gastritis.

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