INFECTION AND IMMUNITY, Oct. 2005, p. 6311–6321 Vol. 73, No. 10
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.10.6311–6321.2005

Major Histocompatibility Complex Class II Inhibits Fas Antigen-Mediated Gastric Mucosal Cell Apoptosis through Actin-Dependent Inhibition of Receptor Aggregation

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Received 2 March 2005/Returned for modification 28 May 2005/Accepted 7 June 2005

Escape from normal apoptotic controls is thought to be essential for the development of cancer. During *Helicobacter pylori* infection, the leading cause of gastric cancer, activation of the Fas antigen (Fas Ag) apoptotic pathway is responsible for early atrophy and tissue loss. As disease progresses, metaplastic and dysplastic glands arise which express Fas Ag but are resistant to apoptosis and are believed to be the precursor cells for adenocarcinoma. In this report, we show that one mechanism of acquired Fas resistance is inhibition of receptor aggregation via a major histocompatibility complex class II (MHCII)-mediated, actin-dependent mechanism. For these studies we used the well-described C57BL/6 mouse model of *Helicobacter pylori* and *Helicobacter felis* infection. Under normal conditions, Fas Ag is expressed at low levels, and MHCII expression on gastric mucosal cells is negligible. With infection and inflammation, both receptors are upregulated, and 6.1% of gastric mucosal cells express MHCII in combination with Fas Ag. Using the rat gastric mucosal cell line RGM-1 transfected with murine Fas Ag and MHCIIαβ chains, we demonstrate that MHCII prevents Fas receptor aggregation and inhibits Fas-mediated signaling through its effects on the actin cytoskeleton. Depolymerization of actin with cytochalasin D allows receptors to aggregate and restores Fas sensitivity. These findings offer one mechanism by which gastric mucosal cells acquire Fas resistance.

Helicobacter pylori is a gram-negative spiral organism which infects over half of the world's population and causes gastroduodenal disease ranging from erosions and ulceration to the most severe manifestation of infection, gastric cancer (24). *Helicobacter pylori* infection is one of the most common chronic infections worldwide and is the causative agent for greater than 80% of gastric cancers (50). The C57BL/6 mouse model of *Helicobacter* infection is a powerful tool with which to study the initiation and progression of gastric cancer. Infection in the C57BL/6 mouse recapitulates human disease, progressing from atrophy and metaplasia through dysplasia to intraepithelial neoplasia with invasion through the muscularis mucosae (9). Using this mouse model to understand the mechanism(s) by which gastric mucosal cells acquires a transformed phenotype will be critical to our efforts to prevent and treat gastric cancer.

Initiation of cancer requires that cells escape from normal apoptotic controls (17). The extension of cell viability through inhibition of apoptosis is a hallmark of human tumors and appears necessary for cells to acquire a transformed phenotype (17). While inhibition of apoptosis per se is not carcinogenic, failure to remove genetically damaged cells which otherwise would have been eliminated allows unchecked accumulation of potentially transforming mutations. *Helicobacter* has been shown to induce apoptosis both directly and indirectly in gastric mucosal cells (6, 11, 14, 36, 37, 41, 43, 44, 46). One apoptotic pathway is the Fas antigen (Fas Ag) signal cascade. Under normal, noninflamed conditions, Fas Ag is expressed at minimal levels throughout the gastric mucosa (51). However, with *Helicobacter* infection there is a marked upregulation of mucosal Fas Ag receptor expression (20, 21), and an increase in Fas Ag expression in both mouse experimental systems and in vitro tissue culture systems results in an increase in apoptotic signaling (20, 22, 26, 29). Fas signaling is crucial for gastric mucosal alterations under inflammatory conditions (21, 26, 33, 38, 52). As the time of infection increases, a subset of cells within the gastric mucosa appear which are resistant to Fas-mediated apoptosis as evidenced by continued survival in the face of persistent Fas Ag expression and available ligand. The Fas Ag-expressing population of cells increases as architectural alterations progress through metaplasia to dysplasia to cancer (28, 51). The mechanism of Fas-apoptosis resistance is not known but has been suggested to include expression of FAP-1, an inhibitor of Fas-mediated apoptosis (27), and FLIP, a dominant-negative caspase 8 (28). In general, Fas-mediated cell death is controlled by an abundance of independent mechanisms allowing cells to integrate a variety of intracellular and extracellular signals to respond in a highly flexible manner towards Fas stimulation. Regulation of the Fas pathway in gastric cells is complex and, at present, incompletely defined.

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Fas Ag is a cell surface receptor. When Fas ligand (Fas L) binds to Fas Ag, receptors aggregate and recruit adaptor proteins, forming the death-inducing signaling complex (DISC) (consisting of Fas L, Fas Ag, FADD and FLICE [procaspase 8]). Once the DISC is formed, a caspase cascade is activated directly (type I signaling) or activated using a mitochondrial amplification loop (type II signaling). Both pathways converge at the level of caspase 3, resulting in apoptosis. There are multiple levels at which this cascade can be regulated, including regulation by parallel signaling pathways and receptor-receptor interactions.

Major histocompatibility complex class II (MHCII) molecules are heterodimeric cell surface glycoproteins whose expression is critical for the development of CD4 T cells and the ability to mount an adaptive immune response. These molecules are expressed on professional antigen-presenting cells, such as B cells, macrophages, and dendritic cells, which function in the uptake, processing, and presentation of antigen to CD4 T cells. In addition, these molecules are constitutively expressed on epithelial cells of the thymus, macrophages, Langerhans cells of the skin (18), and Kupffer cells in the liver (8). Under permissive conditions, MHCII expression can be induced in a variety of cell types, including cells of the gastrointestinal tract (6, 34).

During *Helicobacter* infection, gastric mucosal cells upregulate surface MHCII expression and costimulatory molecules (1, 15, 16, 53) and have the ability to weakly present antigens (1, 47). Additionally, there is evidence that *Helicobacter pylori* may bind the gastric cell via the MHCII complex and regulate apoptotic responses (15). We show that during *Helicobacter* infection there is a small but distinct population of gastric mucosal cells which coexpress Fas Ag and MHCII, setting up a scenario where these receptors may interact, akin to what we see in immune cells. Examination of cells in culture demonstrates that MHCII prevents Fas receptor aggregation at the level of the actin cytoskeleton and renders Fas-expressing gastric mucosal cells resistant to Fas L-mediated apoptosis. These findings support that MHCII coexpression may be one mechanism by which gastric mucosal cells escape Fas-mediated apoptosis.

MATERIALS AND METHODS

Mice. All animal work was approved by the University of Massachusetts Institutional Animal Care and Use Committee. Six- to 8-week-old male C57BL/6 mice which were viral antibody (Ab) free, parasite free, and bacterial pathogen free, inclusive of *Helicobacter* species, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in microisolator cages, fed standard chow, and allowed free access to water.

Bacteria. *Helicobacter felis* (strain 49179 from the American Type Culture Collection, Manassas, VA) and *Helicobacter pylori* Sidney strain (a kind gift from Timothy Wang.) were grown on Trypticase soy agar with 5% sheep blood under microaerophilic conditions at 37° for 4 days. Bacteria were harvested and the number of CFU determined as previously described (22).

Infection with *Helicobacter* species. C57BL/6J mice were restrained by hand and gavaged with *Helicobacter felis* or *H. pylori* (1×10^7 CFU suspended in 400 μl total volume) via a 20-gauge feeding tube (Popper and Sons, New Hyde Park, N.Y.) three times at 2-day intervals. After 6 months of infection, mice were euthanatized, and stomachs were removed, opened along the greater curvature, and washed extensively. The fundic mucosa was gently scraped free from the serosa; minced; digested for 1 h in 1 mM EDTA, 2% bovine serum albumin, and 0.1% pronase in phosphate-buffered saline at 37°C with gentle agitation; filtered through a 40 μm-filter; resuspended in staining buffer with the antibodies CD45-Cy5, Fas-phycoerythrin (Fas-PE), and MHCII-fluorescein isothiocyanate (MHCII-FITC) (BD PharMingen, San Diego, CA); and sorted in different groups.

TABLE 1. Mouse primer sequences

Primer name	Primer sequences (forward and reverse)	Product size (bp)	Annealing temp (°C)
Fas Ag	AAGGGAAGGAGTACATGGACAAGA GAGGCGCAGCGAACACAG	184	55
MHC class II	GACATTGGCCAGTACACATTTGAA TTGGGGAACACAGTCGCTTGAG	230	55
TFF2	GCTTCCCGGGCATCACCAG AAACACCAGGGCACTTCAAA	229	56
GIF	TTGCCCAAATTCTCCCTTCCTT CCCCTCTCAGCTGGTTGTTTATGG	171	55
HK ATPase	CCCCCAATGGCACCTTCAGTCTCC TTCGTAGGGGTCATGGGGGTTGTT	182	59
MUC5	ACGGGGATGGCCACTTTGTTACCT TCTCCGCTTGGCCCTTGCTCTAC	253	59
MUC6	ACTGGCCAGCCTGTCCGAAACT GGGGCCATAAACCATACCATTGAG	255	60
18S rRNA	AAAATAGCCTTCGCCATCACTG GGCTGTACTTCCCATCCTTCAC	217	56

Cytokeratin staining. Fluorescence-activated cell sorter (FACS)-sorted cells were spun onto slides (Thermo Shandon, Pittsburgh, PA) at a speed of 500 rpm for 5 min at a density of 5,000 cells per slide, fixed in 4% paraformaldehyde, pH 7.4, for 1 h, and incubated with the primary Ab for AE1/AE2 keratin complex (DAKO Corporation, Carpinteria, CA) at a dilution of 1/50 using the Animal Research kit (DAKO Corporation, Carpinteria, CA), followed by incubation with diaminobenzidine/hydrogen peroxidase as a chromogen substrate and counterstaining with hematoxylin.

RT-PCR of sorted cells. Populations ($MHCII^+$ $Fas\ Ag^+$ $CD45^-$, $MHCII^+$ $Fas\ Ag^-$ $CD45^-$, and mouse stomach) were subjected to reverse transcription-PCR (RT-PCR) for the following genes: Fas Ag, MHCII, TFF2, gastric intrinsic factor (GIF), hydrogen potassium ATPase (HK ATPase), MUC5, MUC6, and 18S rRNA. RNA was isolated using the RNeasy kit (QIAGEN, Valencia, CA), purified, and DNase treated using a DNA-free RNA kit (Zymo Research, Orange, CA). Reverse transcription was done with the Omniscript RT kit (QIAGEN, Valencia, CA) using the manufacturer's protocol. PCR was done with the HotStarTag Master Mix kit (QIAGEN, Valencia, CA) using the manufacturer's protocol, and the PCR products were run on an agarose gel (2%). RT-PCR was carried out in a programmed thermal Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) using primer sequences and conditions listed in Table 1.

Cell culture. Rat gastric mucosal cells (RGM-1 cell line; RIKEN cell bank, Tsukuba Science City, Japan) were cultured in Dulbecco's modified Eagle medium with 20% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY) at 37°C (with 5% CO_2) and grown to 50 to 70% confluence. Cell lines were cultured as described above with 100 ng/ml rat gamma interferon (IFN-γ) (PeproTech, Rocky Hill, NJ) for 48 h, with 0.5 μg/ml cytochalasin D (CytoD) (Calbiochem, San Diego, CA) for 18 h, and/or with 12.5, 25, or 50 ng/ml human recombinant Fas L for times ranging from 30 min to 48 h (50% lethal dose = 50 ng/ml; Alexis Corporation, San Diego, CA). The parent cell line and cell lines expressing Fas Ag with or without MHCII were exposed to cytochalasin D prior to exposure to Fas L. The dose used in this study (0.5 μg/ml) inhibits actin polymerization without being cytotoxic, as assessed by analytical cytology analysis (40).

Characterization of RGM-1 cells. Total RNA was extracted from RGM1 and MHCII-Fas Ag cells and rat stomach (as a positive control) by using TRIzol (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's directions. Two micrograms of RNA was purified, DNase treated, and reverse transcribed as previously described. Primer sequences for rat TFF2, GIF, TFF2, HK ATPase β subunit, MUC, MUC1, MUC5, MUC6, and 18S rRNA are given

TABLE 2. Rat primer sequences

Primer name	Primer sequences (forward and reverse)	Product size (bp)	Annealing temp (°C)
TFF2	CTGGCGGAAGGCGAGAAAC ATGCCCGGGTATCCACAAT	230	57
GIF	ATTGCCCAGATTCTCCCTTCCT CCCCCTCAGCTGGTTGTGTTTATG	171	55
HK ATPase	TCACGGCGGACATGCTACAGAAT TCCGGGGCTTTTGGGGGTCATC	174	58
MUC	CACCCCCATCTCCACCACCATTAC AGCCCCTCTGAGACATTACACTG	240	56
MUC1	CCAGTGCCGCCGAAAGAGC CAGCCGGGTTGGTGTAAGAGA	191	58
MUC5	CCAAGGGCCTCCACCAACAC TCCAGGCCTGAGCACACACC	201	57
MUC6	ACACCTGTGGGCCCCTCCTACTTG ACTGTGGGCCTTGTGGGTGTTGAC	209	59
18S rRNA	TCCCCGAGAAGTTTCAGCACATCC CTTCCCATCCTTCACGTCCTTCTG	269	59
MHC class II	TCCCTGCGGCGGCTTGAG TCTGACGCTTGTGACGGATGAAAA	406	60
Ii	CCCGATGCGCATGGCTACTCC CCCGCGGCTCTTGGTGTGA	487	60
CIITA	TGCCCACGAAACACAGGAACC GACGGGGCCCAATGCAAACTCTA	466	60
GAPDH	TCTTCACCACCATGGAGAA ACTGTGGTCATGAGTCCTT	231	60

in Table 2. Periodic acid-Schiff (PAS) staining was performed on cytospun cells by using a PAS staining system from Sigma (St. Louis, MO) according to the manufacturer's protocol. Alcian blue staining was performed using alcian blue solution (alcian blue 8GX [Sigma, St. Louis, MO] at 1 g in 100 ml 3% acetic acid, pH 2.5) for 30 min, followed by a tap water wash for 2 min and counterstaining with Nuclear Fast Red (DAKO, Carpinteria, CA) for 5 min. Slides were dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

RGM-1 cells as a model for MHCII signaling in gastric mucosa. Total RNA was extracted from RGM1 cells exposed to 100 ng/ml rat IFN-γ (PeproTech, Rocky Hill, NJ) or control medium and was reverse transcribed and analyzed as previously described, using primers for MHCII RT1B-beta chain, MHCII transactivator (CIITA), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Control reactions were done without the addition of reverse transcriptase.

Fas Ag- and MHCII-expressing clones. RGM-1 cells expressing high levels of surface Fas Ag, MHCII complex alone, both Fas Ag and MHCII complex, or empty vector were made as follows. Total RNA was isolated from mouse thymus and reverse transcribed, and specific sequences were amplified using the following primers: I-A alpha sense, 5′-CTAGCTAGCACCATGCCGCGCAGCAGA GCT-3′; I-A alpha antisense, 5′-CCGGAATTCTCATAAAGGCCCTGGGTG TC-3′; I-A beta sense, 5′-TGCTCTAGAACCATGGCTCTGCAGATCCCCA-3′; and I-A beta antisense, 5′-ATAAGAATGCGGCCGCTCACTGCAGGAG CCCTG-3′. After sequencing (University of Massachusetts Nucleic Acid Facility; ABI Prism model 377 version 3.4.1, ABI200), the 771-bp MHCII α chain was inserted into the NheI/EcoRI site of multiple cloning site A and the 791-bp MHCII β chain was inserted into the XbaI/NotI site of multiple cloning site B of the pIRES cloning vector (Clontech, Palo Alto, CA), and stable clones were selected in neomycin. The 1-kb Fas Ag fragment was cloned using the sense primer 5′-GAAGATCTGCAGACATGCTGTGGATCTGGGCTGTC-3′ and antisense primer 5′-CTCGAATTCTCACTCCAGACATTGTCCTTCATTTTC-3′, sequenced, and inserted into the BglII/EcoRI site of pMSCV-puro (BD Clontech), and stable clones were selected in puromycin. Membrane surface receptor expression was confirmed by FACS analysis (anti-CD95-FITC or MHCII [I-Ak α-FITC and I-Ak β-PE]; PharMingen, San Diego, CA). Expression of MHCII and Fas Ag was analyzed as a percentage of positive cells over background staining. Before these studies, expression of Fas Ag or MHCII on CD45-depleted, *H. felis*-infected (4 to 8 weeks) mouse gastric mucosa was determined by FACS, and populations were classified as high- or low-expressing populations based on surface receptor abundance. The average receptor expression level in the high-abundance population was quantitated relative to GAPDH and 18S rRNA. We then chose our high-Fas-Ag-expressing cell lines and our dual Fas Ag/MHCII cell lines based on the numbers derived from these studies. A cell line was considered a high expressor if it expressed 1.5 to 2.0 times the average level of Fas Ag, or of Fas Ag and MHCII, of the mouse gastric mucosal cells. Three to five representative clones of each group were used to confirm experiments.

Cell growth assays. For proliferation, 1×10^4 cells per well were incubated in 96-well plates with or without 12.5 ng/ml Fas L for 24 h. The plates were harvested and [^{3}H]thymidine incorporation measured as counts per minute. For apoptosis assessment, 1×10^4 cells were seeded into each well of a six-well plate and incubated with or without Fas L at 25 ng/ml for 4 h. Cells were visualized, stained with annexin V and propidium iodide by using an annexin V-FITC kit (Oncogene, Boston, MA) according to the manufacturer's protocol, and analyzed via flow cytometry. Cells that were annexin V positive and PI negative or annexin V positive and PI positive were determined to be apoptotic. Experiments were repeated three times.

Caspase 8 activity assay. Cells (1×10^6) were stimulated for 1 h with Fas L at 25 ng/ml. Stimulated and control cells were collected and analyzed using a Caspase-8 Fluorometric Activity Assay kit (Oncogene, Boston, MA) according to the manufacturer's directions. Caspase 8 activity was measured using a fluorescent plate reader (Perkin-Elmer Life Sciences, Boston, MA) at an excitation wavelength of 400 nm and an emission wavelength of 510 nm. Comparisons were made in relative fluorescence units. Experiments were repeated three times.

DISC immunoprecipitation and analysis. Cells were grown to 70 to 80% confluence and then stimulated with human recombinant FLAG-tagged-Fas L (Alexis Biochemicals) (50 ng/ml) or control vehicle for 30 min. Cells were harvested, washed in cold buffer, pelleted, and lysed in ice-cold lysis buffer (30 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride) with 2 μg/ml small peptide inhibitors (pepstatin, chymostatin, antipain, and leupeptin; Sigma, St. Louis, Missouri) for 30 min. Control cells had FLAG-tagged Fas ligand added after lysis. Cell debris was pelleted by centrifugation for 10 min at $13{,}000 \times g$. FLAG-Fas L-CD95-DISC complexes were immunoprecipitated with an affinity column and anti-FLAG M1 affinity gel (Sigma, St. Louis, MO) according to the manufacture's protocol. The multimeric status of Fas Ag/CD95 was determined by fractionating immunoprecipitated complexes under nonreducing conditions on a 4 to 20% gradient gel (Gradipore Ltd., French Forest, Australia) at 4°C. Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences Corp, Piscataway, NJ) and blocked overnight in 5% nonfat dry milk in 0.1% Tris-buffered saline–Tween 20. Anti-CD95 mouse monoclonal Ab at a 1:2,000 dilution (BD PharMingen, San Diego, CA) was used as the primary Ab, and donkey anti-mouse horseradish peroxidase-conjugated Ab (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:3,000 dilution was used as the secondary Ab. To verify components of DISCs, aliquots were run on reducing gels and blotted using anti-Fas Ag monoclonal antibody at a 1:2,000 dilution (BD PharMingen, San Diego, CA) and rabbit anti-mouse caspase 8 polyclonal antibody at a 1:1,000 dilution (BD PharMingen, San Diego, CA), which detects both noncleaved (inactive) and cleaved forms of caspase 8. Detection was done using the ECL Plus System kit (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's directions.

RESULTS

RGM-1 cells resemble immature gastric mucus cells. Metaplastic and dysplastic cells express both MHCII and Fas Ag and are Fas apoptosis resistant. In order to test the interaction of these two receptors in vitro, we used a cell line derived from mucus cells which resembles cells early in the metaplasia-dysplasia sequence. RGM-1 cells are cytokeratin positive but do not express lineage-specific markers such as GIF (expressed in chief cells), hydrogen potassium ATPase (expressed in parietal cells), MUC5 or MUC6 (expressed in neck and surface epithelial cells), or TFF2 (expressed in metaplasic cells) (Fig. 1A)

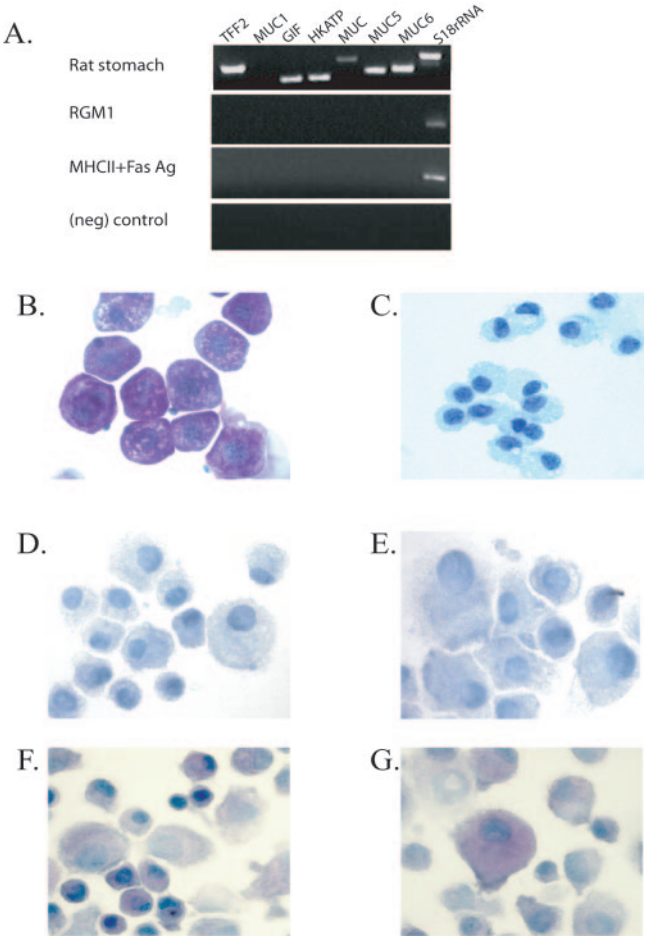

FIG. 1. RGM-1 cells resemble immature mucus cells. (A) RT-PCR for a panel of gastric mucosa-expressed genes in RGM-1 and RGM-1 MHCII/Fas Ag cell lines. Total rat stomach was used as a positive control (top row). RGM-1 cells without reverse transcriptase were used as a negative control (bottom row). (B and C) PAS-hematoxylin staining of primary hepatocyte culture (positive control) (B) and primary lymphocyte culture (negative control) (C). (D and E) Hematoxylin staining of RGM-1 (D) and MHCII/Fas Ag (E) cells. (F and G) PAS-hematoxylin staining of RGM-1 (F) and MHCII/Fas Ag (G) cells.

and do not stain with alcian blue, pH 2.5 (indicative of intestinal type mucin) (data not shown). RGM-1 cells are weakly PAS positive (Fig. 1B to G), indicating glycogen stores.

A population of gastric mucosal cells expresses both Fas Ag and MHCII receptors under conditions of inflammation. During *Helicobacter* infection and inflammation, Fas Ag (21, 22, 26, 33, 38, 52) and MHCII (1, 15, 16, 53) expression on the gastric mucosal cell surface is increased. Representative histological sections from sham-infected (Fig. 2A), *H. felis*-infected (Fig. 2B), and *H. pylori*-infected (Fig. 2C) mice at 6 months show preserved normal architecture in the sham-infected mouse, with a paucity of inflammatory cells and no metaplasia or dysplasia (Fig. 2A). At 6 months (Fig. 2B and C), the chronically infected mice have developed moderate submucosal plasma-lymphocytic infiltrates, with lymphocytes invading the mucosal layer and intercalating between glandular units. There is a decrease in the number of parietal and chief cells, antralization of glands and early metaplasia. There was no

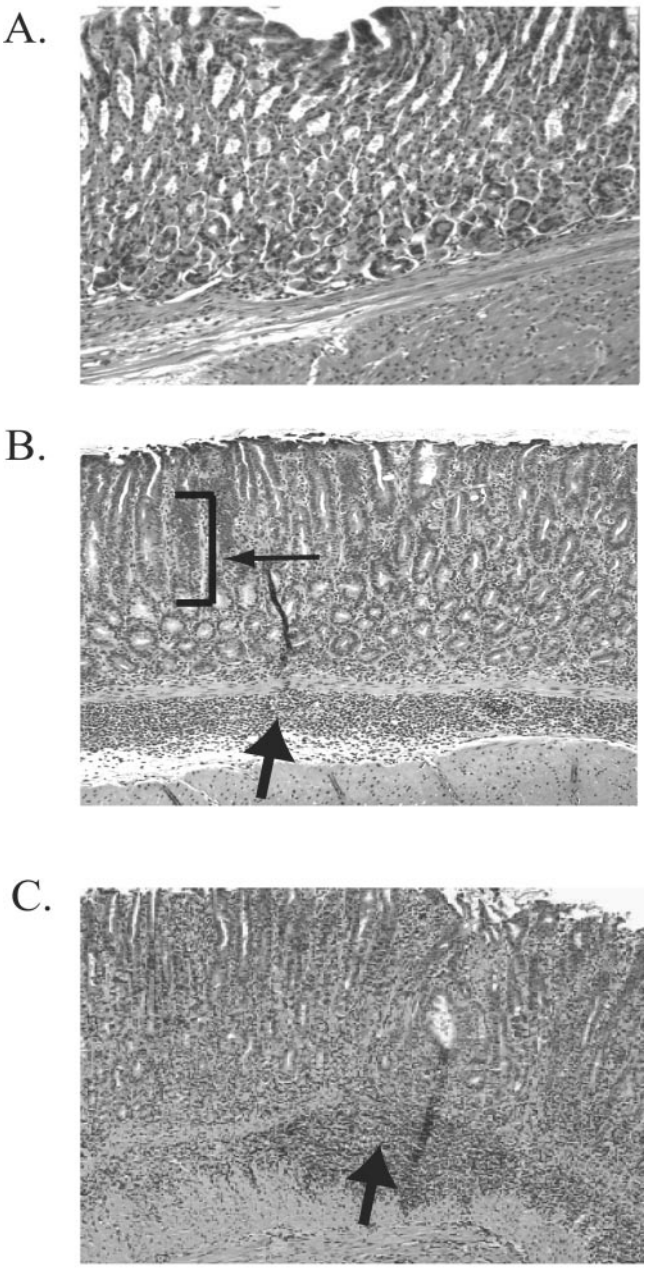

FIG. 2. Histological changes due to *Helicobacter* infection. Six- to 8-week-old male C57BL/6 mice were infected with control medium (sham infected), *H. felis*, or *H. pylori* and euthanized at 6 months. The gastric mucosa of sham-infected mice (A) had normal architecture, with preservation of parietal cells and a paucity of inflammatory cells. The gastric mucosa of mice infected with *H. felis* (B) or *H. pylori* (C) had antralization of glands (thin arrow), loss of parietal cells, and chronic inflammatory infiltrate in the submucosa (thick arrows) and above the muscularis mucosa, between glands. Hematoxylin and eosin staining was used; magnification, ×400.

evidence of dysplasia at this early time point. The findings were similar for both the *H. felis*- and *H. pylori*-infected groups; however, the range of mucosal alterations was broader with *H. pylori* infection. *H. felis* infection, which showed less mouse-to-

mouse variation, was used for the remainder of the experiments.

To determine both the percentage of cells expressing Fas Ag and MHCII and whether expression occurred within the same gastric mucosal cell, we examined single-cell preparations of the gastric mucosa that had been depleted of immune cells ($CD45^+$) by FACS. Under control conditions, MHCII is expressed on a small minority of gastric mucosal cells, and only 0.12% of cells coexpressed Fas Ag and MHCII. In contrast, mice infected with *Helicobacter felis* expressed both MHCII and Fas Ag on the surface of greater than 6% of the gastric mucosal cells isolated (representative data from one experiment are shown in Fig. 3A). Cytokeratin staining confirmed that these dual-receptor-expressing cells were epithelial cells (Fig. 3B and C).

We further determined the nature of these dual-receptor-expressing cells. We analyzed whole gastric mucosa (containing all gastric cell lineages and inflammatory cells) and two populations of CD45-negative gastric cells, a double-positive population expressing both Fas Ag and MHCII and a double-negative population which expressed neither Fas Ag or MHCII, for the expression of lineage specific markers. Fas Ag and MHCII are coexpressed on cells expressing a wide range of lineage markers, suggesting that this double-receptor-expressing population is a heterogeneous mixture of parietal, chief, mucous, and metaplastic cells (Fig. 3D).

Gastric mucosal cells express MHCII in response to IFN-γ. In order to study MHCII/Fas Ag signal interactions, we turned to a tissue culture system. Rat gastric mucosal (RGM-1) cells in culture do not express MHCII or costimulatory molecules under normal culture conditions (Fig. 4A, lane 1). Upon exposure to IFN-γ, MHCII and invariant chain (Ii) expression and CIITA are markedly increased at both the mRNA (Fig. 4A, lane 2) and protein (Fig. 4B) levels. Control experiments were done without reverse transcriptase enzyme to exclude genomic DNA sequence amplification (Fig. 4A, lanes 3 and 4). RGM-1 cells cultured with IFN-γ for 24 to 72 h elongate and develop an increased number of cytoplasmic projections (Fig. 4C and D). RGM-1 cells express surface Fas Ag after exposure to the inflammatory cytokines IL-1β, TNF-α, and IFN-γ (22), similar to what is seen in vivo, further supporting the use of this cell line as a model for MHCII/Fas interaction in gastric mucosal cells.

Gastric cell lines expressing MHCII have altered morphology and growth characteristics. In order to address potential interactions between MHCII and Fas Ag in gastric mucosal cells, while avoiding potentially confounding effects of exogenous IFN-γ, we established cell lines stably expressing the MHCII complex and the Fas Ag receptor. FACS analysis confirmed surface expression of both the MHCII alpha and beta chains (Fig. 5A and B) and Fas Ag (Fig. 5C). Clones which expressed high physiological levels (see Materials and Methods) of both receptors were chosen for further study. In culture, RGM-1 cells have a hexagonal shape and rarely show cell projections (Fig. 3C). RGM-1 cells transfected with empty vector or those expressing high levels of Fas Ag alone did not differ in size, shape, or growth characteristics from the control cell line (data not shown). Interestingly, cell lines expressing MHCII alone or MHCII/Fas Ag (Fig. 5D) had a flattened, fusiform, spindle-shaped phenotype with long cytoplasmic projections and were phenotypically indistinguishable from RGM-1 cells exposed to IFN-γ (Fig. 4D). There was no increase in spontaneous apoptosis or cell death in any of the cell lines.

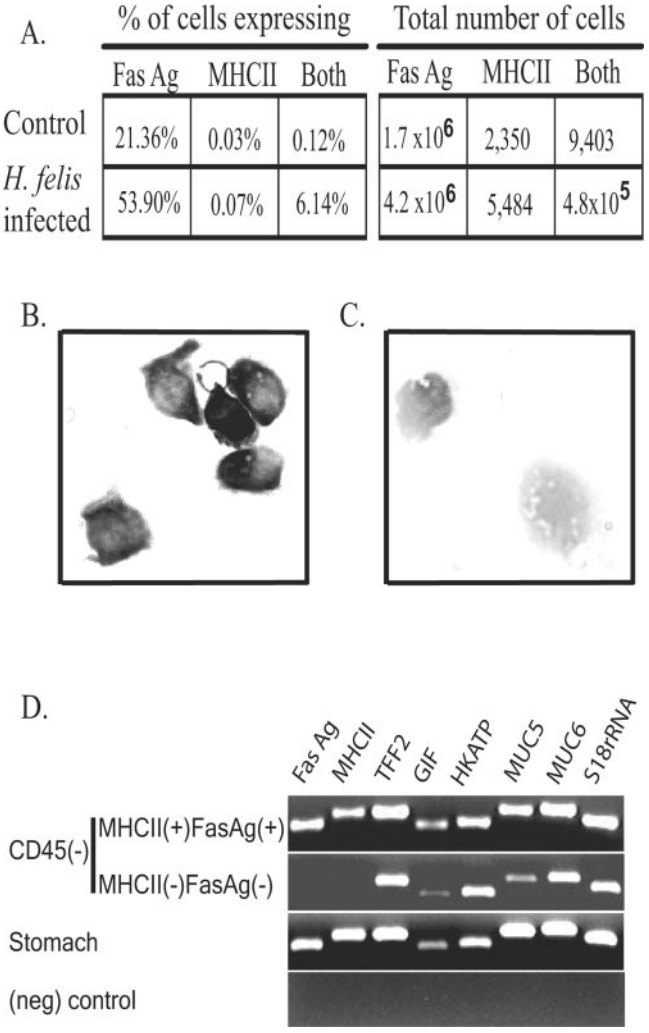

FIG. 3. Fas Ag and MHCII are coexpressed on gastric epithelial cells during *Helicobacter* infection. (A) Single-cell preparations from control or *H. felis*-infected mice were stained with CD45-Cy5, Fas Ag-PE, and MHCII-FITC and FACS sorted into $CD45^-$ populations expressing Fas Ag, MHCII, or Fas Ag/MHCII. Results of one representative experiment are shown. The experiment was performed three times with similar results. Cells from mice infected with *H. pylori* gave a similar pattern of expression, and results are not shown. (B and C) Cytokeratin staining of $CD45^-$ MHCII/Fas Ag^+ (B) and $CD45^+$ (C) populations confirms that the $CD45^-$ MHCII/Fas Ag^+ are of an epithelial nature. (D) RT-PCR for expression of a panel of lineage-specific genes. The $CD45^-$ population was sorted into MHCII/Fas Ag-expressing (top row) and non-MHCII/Fas Ag-expressing cells (second row) populations. cDNA prepared from total mouse stomach was used for controls. Third row, positive control; fourth row, without reverse transcriptase, negative control.

Gastric mucosal cells expressing MHCII are resistant to Fas-mediated cell signaling. In culture, RGM-1 cells express negligible Fas Ag surface receptor; have a low level of spontaneous apoptosis, and have a very small increase in apoptosis in response to Fas L (Fig. 6A and B). RGM-1 cells expressing

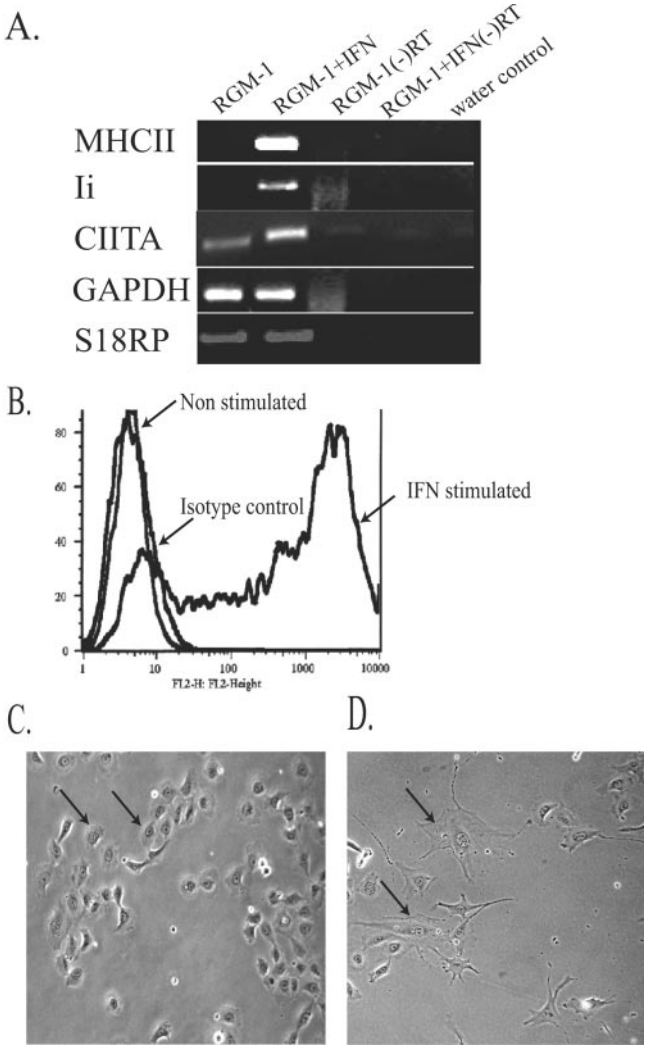

FIG. 4. IFN-γ regulates MHCII expression in gastric mucosal cells. Rat gastric mucosal cells (RGM-1) were cultured in the absence or presence of 100 ng/ml rat IFN-γ for 24 to 72 h. (A) RT-PCR for MHCII, Ii, and CIITA expression after 24 h in culture. GAPDH and S18 ribosomal message were used to standardize the blots. (B) Surface MHCII expression was confirmed by FACS analysis. (C) Cells grown for 72 h in control medium have a compact hexagonal shape (arrows). (D) Cells grown with 100 ng/ml IFN-γ for 72 h are elongated with stellate projections (arrows).

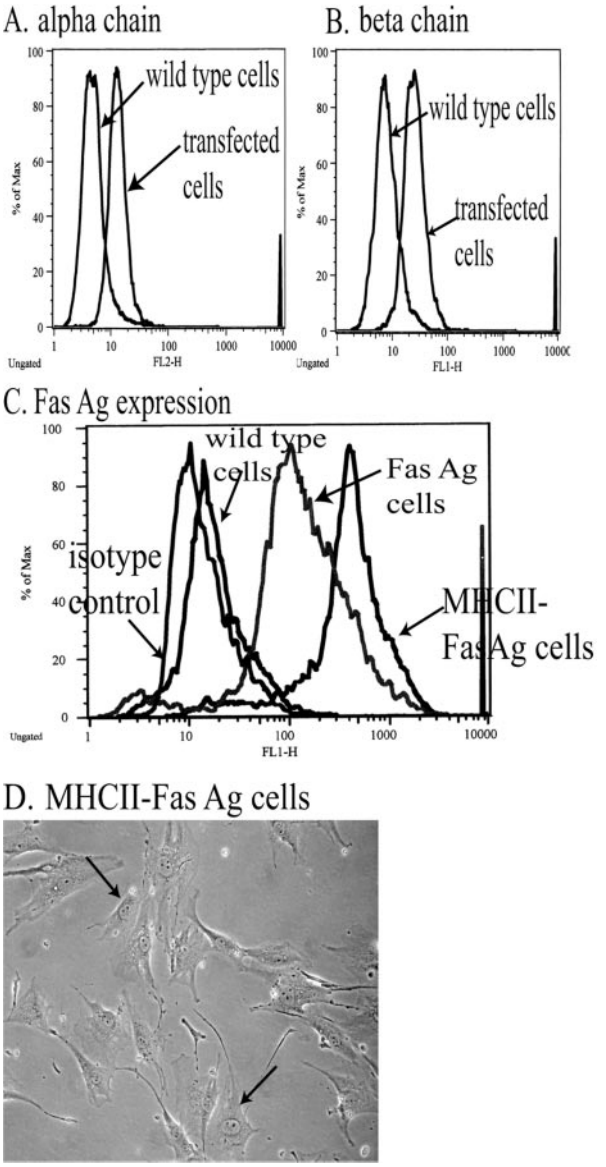

FIG. 5. Morphological alterations of gastric mucosal cells expressing both MHCII and Fas Ag. RGM-1 cells were transfected with appropriate expression vectors. (A to C) Expression of alpha chain (A), beta chain (B), and Fas Ag (C) surface receptors was confirmed by FACS analysis. (D) Cellular morphology after 72 h in culture. Arrows show long stellate projections.

MHCII on the cell surface also have a low baseline level of apoptosis which is not increased with the addition of Fas L (Fig. 6C and D). In sharp contrast, RGM-1 cells expressing abundant Fas Ag on the cell surface rapidly undergo apoptosis in response to the addition of 25.0 ng/ml Fas L, with 38% ± 5% of cells being apoptotic at 4 h (Fig. 6E and F) and greater than 98% being apoptotic at 24 h (data not shown). Strikingly, cells expressing both MHCII and Fas Ag are completely resistant to Fas L-induced apoptosis (Fig. 6G and H). Dual-receptor-expressing cells did not undergo apoptosis in response to 25.0 ng/ml Fas L for 4 or 24 h (Fig. 6G and H; summarized in 6I) and showed no increase in caspase 8 activity (Fig. 6J), suggesting that the block to signaling was upstream of caspase activation.

Gastric mucosal cells can use the Fas Ag pathway for proliferative signaling at low receptor stimulation (due to low receptor abundance or low ligand level) (22, 29) and for both proliferative and apoptotic signaling at high receptor stimulation (22, 29, 30). We next tested whether Fas proliferative signaling was intact in cells which coexpress MHCII and Fas Ag. Consistent with our previous reports, RGM-1 cells expressing low-abundance Fas receptor exposed to low-level Fas L (6.25 or 12.5 ng/ml) significantly increased proliferation ($P < 0.01$) compared with cells exposed to control culture conditions. Cells expressing both MHCII and high levels of surface Fas Ag had no growth response to ligand. Dual-receptor-ex-

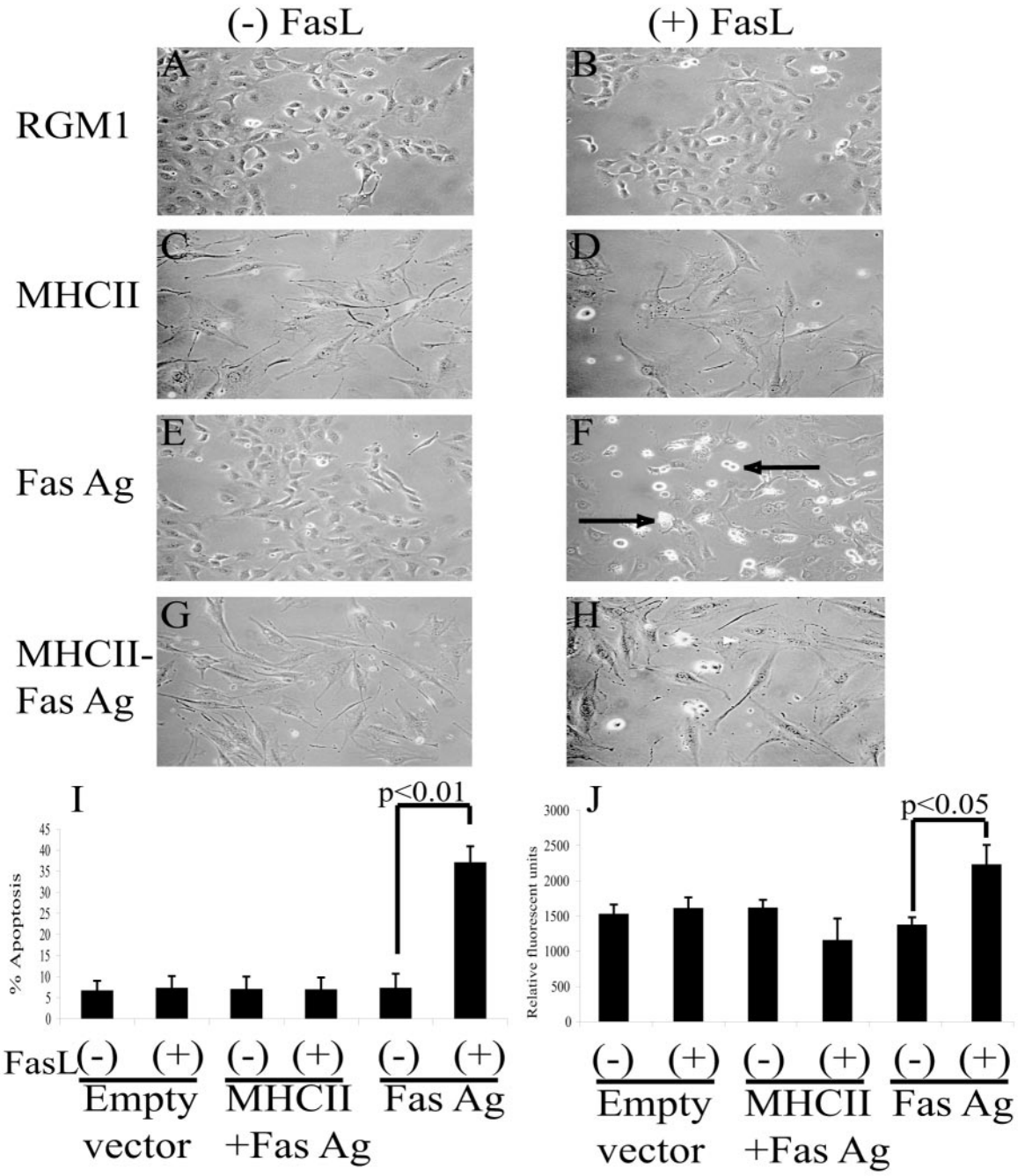

FIG. 6. Gastric mucosal cells expressing MHCII and Fas Ag are resistant to Fas L-induced apoptosis. Cells were exposed to control medium or medium containing 25 ng/ml Fas L for 4 h. (A and B) RGM-1 cells have a low basal level of apoptosis and do not undergo morphological changes of apoptosis when exposed to ligand. (C and D) Cells expressing MHCII do not undergo apoptosis under control conditions or upon exposure to Fas L. (E and F) Cells expressing Fas Ag do not undergo spontaneous apoptosis but show rounding, detachment, and blebbing (arrows) upon addition of ligand. (G and H) Cells with a higher level of Fas Ag expression as than in panels E and F but also expressing MHCII do not undergo spontaneous apoptosis and do not increase apoptosis in response to ligand. (I) Levels of apoptosis were quantitated using annexin V staining in cell lines with or without 25 ng/ml Fas ligand for 4 h as indicated. (J) Cells were grown under the same culture conditions as for panel I for 1 h, and caspase 8 activity was measured using the Caspase-8 Fluorometric Activity Assay kit. All experiments were repeated three times. Results in panels I and J are averages from three experiments ± 1 standard deviation.

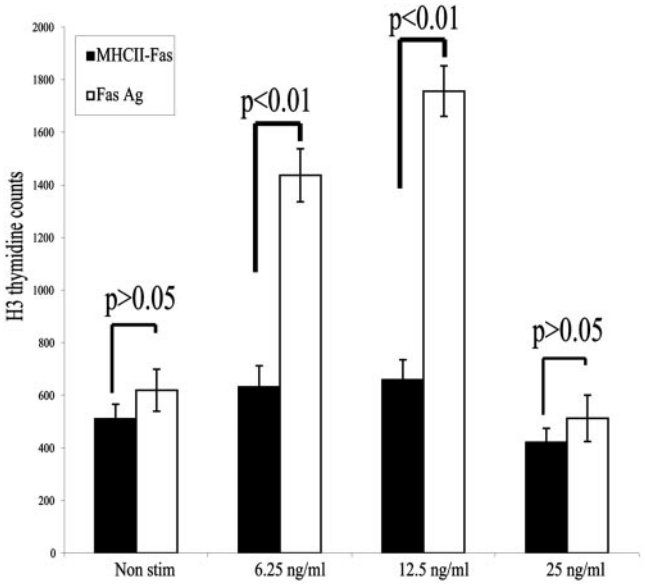

FIG. 7. MHCII coexpression inhibits Fas-mediated proliferative signaling, RGM-1 cells expressing MHCII and Fas Ag or Fas Ag alone were cultured for 24 h in control medium or medium containing 6.25 to 25 ng/ml Fas ligand (as indicated), and proliferation was measured by [^{3}H]thymidine incorporation. Experiments were repeated three times, and results represent the averages ± 1 standard deviation.

pressing cells had very low baseline levels of proliferation as measured by [^{3}H]thymidine incorporation, which did not change with the addition of 6.25 to 25 ng/ml Fas L (Fig. 7), suggesting that all Fas-mediated growth signaling was interrupted in these cells.

MHCII inhibits Fas signaling by preventing receptor aggregation. In order to determine the mechanism of Fas resistance, we first determined the point within the pathway where regulation likely occurred. Caspase 8 is not activated in dual-receptor-expressing cells; therefore, MHCII interferes with Fas signaling at or above the level of caspase 8 activation (Fig. 6J). Coexpressing cells avidly bind Fas L-specific antibody (Fig. 5B), and therefore we reasoned that the defect occurred at the level of signal propagation below receptor ligation. Levels of the inhibitor FLIP were unchanged between cell lines (data not shown). Because of the alteration in cell morphology with MHCII, we addressed whether receptor aggregation was impaired. FLAG-tagged Fas L was used to immunoprecipitate DISCs (Fas L, Fas Ag, FADD, and FLICE) from Fas Ag-expressing and Fas Ag/MHCII-expressing cell lines. After the addition of ligand to Fas Ag-expressing cells (Fig. 8A, lane 3), only signal-competent 250-kDa multimeric complexes were isolated (Fig. 8A, top gel). These bands were confirmed to be specific for DISCs by running an aliquot on a denaturing gel and blotting with Fas Ag (Fig. 8A, middle gel), which produced only a single 48-kDa band for each sample, or caspase 8 (Fig. 8A, bottom gel). Strikingly, in cells expressing both Fas Ag and MHCII, a significant portion of receptors remained as 75-kDa monomeric complexes and failed to aggregate (Fig. 8A, lane 1); this failure to aggregate could not be overcome with either longer incubation times or higher levels of ligand (data not shown). Scant uncleaved caspase 8 (50 to 55 kDa) was recovered from these DISCs (Fig. 8A, third gel, lane 1). Inhibition of actin polymerization by CytoD completely restored receptor clustering, as only aggregated receptor complexes were recovered (Fig. 6A, lane 2). In addition, abundant caspase 8 was recovered in these DISCs and existed as both uncleaved (50- to 55-kDa) and active (41- to 43-kDa) forms, comparable to that which was detected in Fas Ag-only expressing cells (lane 3). In the presence of CytoD, MHCII/Fas Ag-expressing cells lost their stellate cytoplasmic projections and assumed a more hexagonal shape, similar to wild-type control cells (data not shown). With the addition of ligand, caspase 8 activation was restored to the same level as seen in cells expressing Fas Ag only (Fig. 8B), and the addition of CytoD restored the Fas L-induced apoptotic response (Fig. 8C) while not inducing an increase in spontaneous death in the absence of ligand. CytoD had no effect on the basal level or Fas L-mediated apoptosis in the parent RGM-1 cell line or in cells expressing high levels of Fas Ag alone (Fig. 8C).

DISCUSSION

Dysregulation in the balance between proliferation and apoptosis is felt to underlie the development of cancer; it is not that inhibition of apoptosis per se is carcinogenic, but rather that inhibition of apoptosis in cells which otherwise would have been eliminated increases the likelihood of accumulating harmful genetic defects, leading to a proliferative advantage and the emergence of neoplastic clones (12, 17). In the setting of normal inflammation, that is, inflammation associated with acute injury and wound healing, environmental alteration due to chemokines and cytokines is a self-limited event (12). With chronic inflammation, such as is seen with *Helicobacter* infection, there is constant exposure of the mucosa to cytokines and chemokines, leading to dysregulation of growth (13, 42, 50). Growth programs for repair and proliferation are activated, and apoptotic programs may be bypassed in an attempt to restore mucosal integrity. This sedition of the normal cell death and repair programs results in abnormal DNA replication and proliferation, pushing cells to acquire mutations (12). Because Fas-mediated apoptosis is a central feature of cell loss in *Helicobacter* infection (1, 13, 15, 16, 19, 21–25, 33, 39, 42, 45, 50, 52), alterations in the regulation of this pathway have great potential for contributing to the malignant process.

Here we show that one mechanism of apoptosis escape in the inflamed stomach is through the acquisition of MHCII expression and its subsequent interaction with Fas Ag signaling cascade.

Fas Ag signaling is well recognized as a mediator of gastric mucosal apoptosis and plays a central role in *Helicobacter*-induced mucosal injury (21, 23, 25, 26, 38, 52). In the face of inflammation, Fas Ag is highly expressed in numerous cell types within the gastric mucosa, including invading inflammatory cells and gastric epithelial cells, with the highest expression seen in chief and parietal cell compartments (38). Certainly, surface Fas Ag appears to be necessary for parietal cell loss and atrophy (21, 33, 38), which are early and progressive events in *Helicobacter* disease (21), culminating in gastric mucosal atrophy. In the setting of atrophy, there is peripheral stem cell failure and an influx of bone marrow-derived stem cells in an apparent reparative effort (16). These marrow-

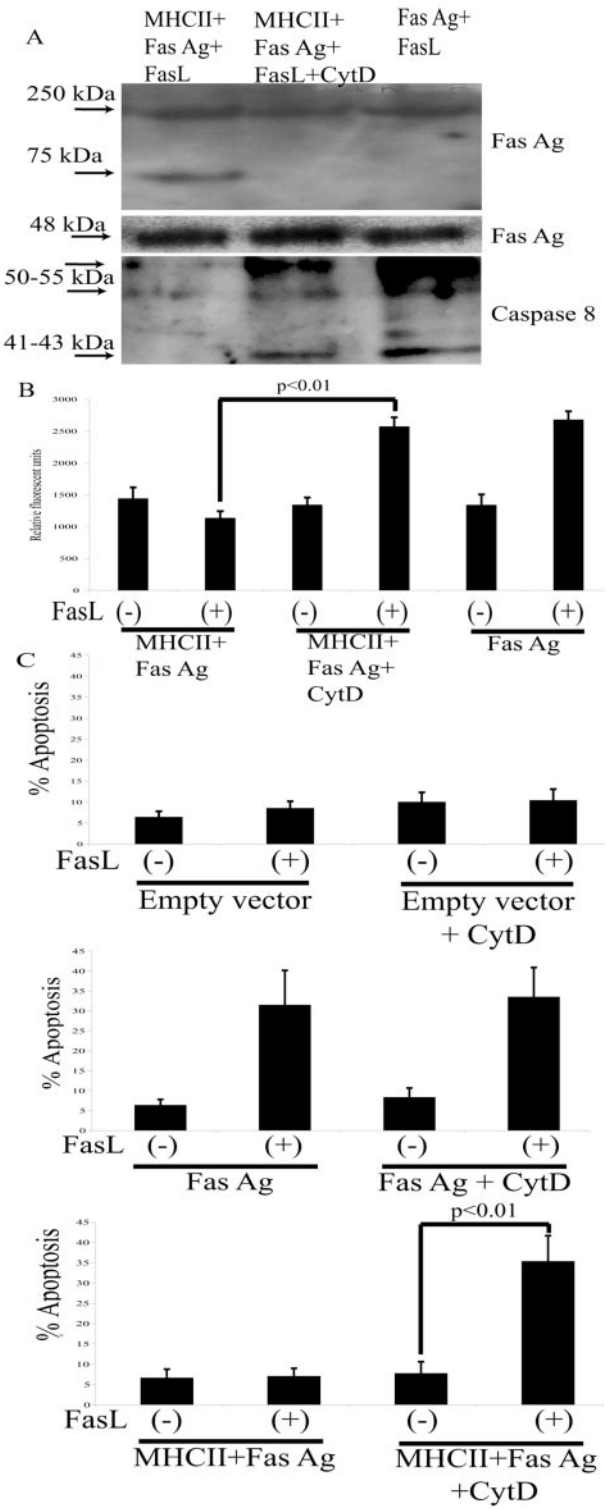

FIG. 8. MHCII inhibits Fas Ag signaling through an actin-dependent inhibition of receptor aggregation. (A) Cells were cultured with FLAG-tagged Fas L at 25 ng/ml for 15 min. DISCs were isolated from MHCII/Fas Ag-expressing cells (lane 1), MHCII/Fas Ag-expressing cells grown in the presence of cytochalasin D at 0.5 μg/ml to inhibit actin polymerization (lane 2), or Fas Ag-expressing cells (lane 3). Proteins were run on a nonreducing gel and blotted with anti-Fas Ag antibody. Monomeric complexes are seen at 75 kDa, and aggregated complexes are seen at 250 kDa. Bands were confirmed to be specific for Fas aggregates by running an aliquot of the sample on a reducing gel and blotting for Fas Ag (middle gel) or caspase 8 (bottom gel). (B) Cell lines were grown in the presence or absence of Fas L at 25 ng/ml for 1 h as indicated, and caspase 8 activity was measured and is presented as relative fluorescent units. Bars represent averages from three experiments ± 1 standard deviations. (C) Control cells (empty vector) (top panel), Fas Ag-expressing cells (middle panel), and MHCII/Fas Ag-expressing cells (bottom panel) were grown in the presence or absence of cytochalasin D, with or without Fas L at 25 ng/ml, for 4 h as indicated, and apoptosis was measured by annexin V staining. Each bar represents the average from three experiments ± 1 standard deviation.

derived cells engraft and differentiate toward an incomplete gastric cell phenotype, forming the basis of metaplastic and dysplastic glands. While most cells expressing high receptor abundance (specifically parietal and chief cells) undergo apoptosis early in infection (13, 38), these metaplastic and dysplastic cell appear to be resistant to apoptotic signaling (27, 28). This phenotype of apoptosis resistance in the setting of high receptor abundance suggests that a mechanism other than receptor number is used to regulate susceptibility to Fas signaling events in these cells and may involve several points of regulation along the Fas signaling cascade.

While much is known about Fas signaling in immune cells, much less is known about signaling regulation in other cell types. In general, Fas-mediated cell signaling events are controlled by an abundance of independent mechanisms, including but not limited to factors which alter the expression level and location of Fas Ag, availability of ligand, assembly and function of the DISC, and regulation of caspase activation and function. Integration of a wide variety of extracellular and intracellular events in the context of cell type specificity confers a high degree of flexibility to Fas L/Fas Ag signaling outcomes. We have previously shown that one level of regulation of the Fas pathway within the gastric mucosa is through modulation of receptor number. High levels of IL-1β, TNF-α, and IFN-γ, found in the infected gastric mucosa, regulate surface Fas Ag expression (22) and are associated with more severe diseases states (13, 42, 45), due in part to increased Fas signaling. Interestingly, this same environment which increases Fas Ag expression (most notably, elevated IFN-γ levels) regulates MHCII expression in gastric mucosal cells, albeit with different levels of protein expressed in different cell types. This differential expression of Fas Ag and MHCII within the gastric mucosa creates the potential for receptor interaction and variable signaling outcomes. MHCII is expressed in metaplastic and dysplastic tissue (unpublished observation) as well as in carcinoma (1, 7, 48), making a scenario of MHCII/Fas Ag receptor interaction plausible.

As is true with other aspects of Fas signaling, the interaction between the MHCII complex and the Fas Ag pathway is cell type specific and appears to be unique to each situation studied. For example, in lymphoid cells, MHCII expression paradoxically increases Fas-mediated signaling in B lymphocytes, where MHCII expression is a cofactor in B-cell-mediated apoptosis through the production of "second messengers" (49), the identities of which are incompletely defined (5). Quite the opposite, MHCII ligation via interaction with T cells confers rapid Fas resistance in B cells (32) and in T cells, where CD4

and MHCII interactions are required for survival of resting CD4 cells; coexpression offers resistance against Fas-mediated apoptosis (10, 35). Immature dendritic cells, which express moderate levels of Fas surface receptor and low levels of or absent MHCII, are highly sensitive to Fas-mediated apoptosis. As these cells mature and express high levels of MHCII, they become fully resistant to Fas apoptotic signaling (35), while maintaining sensitivity to MHCII-induced apoptosis. Because gastric mucosal cells acquire many characteristics of immune cells during *Helicobacter* infection, including cytokine production (31) and the capacity to present antigens (4), and of cells derived from the marrow, which engraft and differentiate towards a gastric phenotype yet may maintain characteristics of marrow cells, we addressed whether cells within the infected gastric epithelium possess MHCII/Fas Ag interactions similar to those found in lymphocytes and/or dendritic cells. In this report, we show that MHCII molecules inhibit Fas-mediated signaling in gastric mucosal cells through inhibition of receptor aggregation and prevention of downstream caspase activation by an actin cytoskeleton-dependent mechanism.

In our gastric epithelial cell model, expression of MHCII alone or in combination with Fas Ag alters the actin cytoskeletal arrangement; cells assume a spindloid, fusiform morphology with prominent projections, which is reversed with inhibition of actin polymerization (cytochalasin D). Short-term cytochalasin D treatment effectively restored the normal hexagonal shape without increasing baseline apoptosis or necrosis and effectively restored susceptibility to Fas-mediated apoptosis by allowing receptor clustering (Fig. 8A).

Under conditions of self-limited inflammation, MHCII expression by gastric mucosal cells may be beneficial. Indeed, presentation of antigens (3) by gastric mucosal cells and binding of *Helicobacter* organisms directly to the MHCII complex (15, 16) have been reported and potentially play a role in coordinating the local immune response to infection. However, the immune response is ineffective, and failure to eliminate the organism results in a continued inflammatory state. Environmental conditions supporting MHCII expression on normal gastric mucosal cells also appear to increase expression on other populations, such as the bone marrow-derived metaplastic and dysplastic populations. Under these conditions, MHCII expression may become detrimental. The inhibition of apoptosis due to MHCII involvement may be one mechanism by which abnormal clones of cells avoid apoptosis and may help explain the progression of the metaplastic cell lineage to adenocarcinoma.

Retrospective studies suggest that differences in patient susceptibility to gastric cancer may be linked to the MHC locus (for example, expression of the MHC DQA1*0102 allele is associated with a decreased risk of intestinal-type gastric adenocarcinoma [2]), offering the exciting possibility of further identifying the genetic compositions of patients most at risk for gastric cancer and offering targeted intervention based on these signaling alterations.

ACKNOWLEDGMENTS

This work was supported by NIH grant K22 CA90518 and American Cancer Society institutional research grant IRG-93-033 (to J.H.)

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Editor: J. D. Clements