Proteasome Subunit LMP2 is Required for Matrix Metalloproteinase-2 and -9 Expression and Activities in Human Invasive Extravillous Trophoblast Cell Line

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The ubiquitin-proteasome pathway (UPP) is involved in the degradation of the extracellular matrix (ECM) and trophoblastic invasion during early pregnancy. Our previous studies demonstrated that inhibition of UPP suppresses expression of matrix metalloproteinase (MMP)-2 and -9. LMP2 is an important proteasome subunit that is critical for proteasome activity. This study investigated the regulatory mechanism of LMP2 on the expression and activities of MMP-2 and MMP-9. Our results showed that transfection of LMP2 siRNA plasmid into the human invasive extravillous trophoblast cell line (HTR8/Svneo) could significantly suppress expression of LMP2 mRNA and protein. The mRNA expression of MMP-2 and MMP-9 and their activities were markedly decreased in the LMP2-inhibited cells. Inhibition of LMP2 could also reduce IκBα mRNA level, although the expression of phosphorylated IκBα was increased. In the LMP2-inhibited cells, expression of mRNA encoding NF-κB subunits p50 and p65 remained normal, but the p50 protein level was significantly decreased in the cytosolic and nuclear extracts, while p65 protein was markedly reduced only in the nuclear extract. We also demonstrated that blockage of the NF-κB pathway by the NF-κB translocation inhibitor SN50 markedly reduced the expression of MMP-2 and MMP-9 in HTR8/Svneo cells, a result that is fully consistent with the results from the LMP2-inhibited HTR8/Svneo cells. These data suggest that LMP2 contributes to IκBα degradation and p50 generation, and that inhibition of LMP2 suppresses expression and activities of MMP-2 and MMP-9 by blocking the transfer of active NF-κB heterodimers into the nucleus. J. Cell. Physiol. 206: 616–623, 2006. © 2005 Wiley-Liss, Inc.

The 26S proteasome is the main protease in the cytoplasm and nucleus of eukaryotic cells, which recognizes and degrades ubiquitinated proteins, including misfolded or damaged, and other regulatory proteins (Reits et al., 1997; Voges et al., 1999). The proteolytic core complex of the 26S proteasome is the 20S proteasome, which consists of 28 subunits arranged in four stacked heptameric rings, with the outer two rings containing seven different α-type subunits and the inner two rings containing seven different β-type subunits (Lowe et al., 1995; Groll et al., 1997). Each proteolytic β-type subunit expressed with an NH_2-terminal threonine residue is critical for multiple peptidase activities, including chymotryptic-, tryptic-, and peptidylglutamyl-like activities (Schmidtke et al., 1996; Baumeister et al., 1998). LMP2 is an important subunit with an NH_2-terminal threonine residue in the inner rings (Kuchelkorn et al., 1995). Several experiments show that presence of LMP2 in the 20S proteasome results in increased chymotryptic and tryptic activities in vitro, and modulates the cleavage site preferences of the proteasome (Ustrell et al., 1995; Gaczynska et al., 1996). LMP2 knockout mice exhibit a defect in proteasome function (Hayashi and Faustman, 2002). The UPP is involved in the regulation of cell cycle, degradation of some transcription factors, modification of some membrane proteins, assembly of ribosomes, and antigen presentation of major histocompatibility complex class I (MHC-I) (Craiu et al., 1997; Ciechanover, 1998). Our previous study showed that the proteasome subunit LMP2 is strongly expressed in the trophoblastic cells of placental villi and plays important roles in the degradation of the extracellular matrix (ECM) and trophoblast invasion during early pregnancy (Wang et al., 2004b).

Matrix metalloproteinases (MMPs), a family of endopeptidases with the ability to degrade ECM proteins, play a fundamental role in inflammation, tissue remodeling, angiogenesis, wound healing, tumor invasion, and metastatic progression (Nagase and Woessner, 1999; McCawley and Matrisian, 2001). Among the MMPs, MMP-2 and MMP-9 are key enzymes synthesized as latent proenzymes, which must be activated in order to show their proteolytic activities and degrade various components of the ECM including type IV, V, VII, and X collagens, fibronectin, and gelatin (Allan et al., 1995; Nagase and Woessner, 1999; Xie et al., 2004). MMP-2 and MMP-9 have been extensively

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investigated because of their recognized roles in early pregnancy (Shah and Catt, 2004). It has been demonstrated that MMP-2 and MMP-9 play key roles in ECM degradation and trophoblast invasion during early pregnancy (Polette et al., 1994; Xu et al., 2002). We have also documented that the UPP is involved in regulating MMP-2 and MMP-9 mRNA expression and the gelatinolytic activity of MMP-9 (Wang et al., 2004b), however, there is still no direct evidence to demonstrate that proteasome subunit LMP2 is involved in regulating MMP-2 and MMP-9.

Many reports demonstrated that some signal pathways are involved in regulating MMP-2 and MMP-9 expression. NF-κB pathway is just one of the important factors. SN50, a peptide inhibitor of NF-κB translocation, inhibits MMP-2 and MMP-9 expression and activity to varying degree in different cell lines (Han et al., 2001; Hozumi et al., 2001). Five members of the NF-κB family, p50, p52, p65 (RelA), c-Rel, and RelB, form various homodimers and heterodimers, where the most common and active NF-κB heterodimer is composed of the p50 and p65 subunits (Hayden and Ghosh, 2004). The NF-κB heterodimer is generally associated with a member of the IκB family in the cytosol (Yamamoto and Gaynor, 2004). Moreover, it is also known that the UPP is responsible for the generation of p50 and p52, thereby allowing them to form heterodimers with p65 (Lin et al., 1998; Sears et al., 1998). Cellular stimulation results in the phosphorylation and subsequent proteolytic degradation of phosphorylated IκBα, which allows NF-κB to enter the nucleus where it then regulates the expression of its target genes (Chen et al., 1995). Although it has been reported that LMP2 is required for the generation and activation of NF-κB in human lymphocytes that lack LMP2 gene (Hayashi and Faustman, 2000), little is known about the essential role of LMP2 in regulating IκBα and NF-κB expression in normal human cell lines. Moreover, whether LMP2 regulates the expression and activities of MMP-2 and MMP-9 via the NF-κB pathway, is still unclear.

The aim of this present study was to investigate the regulatory role of LMP2 in the expression and activities of MMP-2 and MMP-9 using RNAi, and to determine if these effects are realized via the NF-κB pathway.

MATERIALS AND METHODS

Cell culture and treatment

The human invasive extravillous trophoblast cell line (HTR8/Svneo) was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and a 1% penicillin/streptomycin mixture (Invitrogen Corp., Gaithersburg, MD). The cells were cultured in 95% air and 5% CO_2 at 37°C. The peptide inhibitor for NF-κB, SN50 (Calbiochem Inc., La Jolla, CA), was dissolved in DMSO (Sigma, St. Louis, MO) followed by dilution with RPMI 1640 medium without fetal bovine serum. The final concentration of SN50 was 50 μg/ml. Medium without fetal bovine serum was harvested for zymography, and cells were collected for total RNA extraction after 24 h incubation.

Plasmid construct and transfection

The pSilencer™ (2.1-U6 hygro) siRNA expression vector was purchased from Ambion (Ambion Inc., Austin, TX). The specific sequences of the LMP2 hairpin siRNA inserts are as follows: 5′-GATCCATATCGAGAGGACTTGTCTTTCAAGAGAAGACAAGT CCTCTCGATATTTTTTTGGAAA-3′ (forward); 5′-AGCTTTTCCAAAAAAATATCG AGAGGACTTGTCTTCTCTTGAAAGACAAGTCCTCTCGATATG-3′ (reverse). The hairpin LMP2 oligonucleotides were annealed and ligated into the vector according to appropriate protocols. The negative control siRNA plasmid, whose inserted fragment has no significant homology to mouse, rat, or human gene sequences, was used in this study as a control for non-specific effects on gene expression. Plasmids were purified using the EndoFree Plasmid Kit (QIAGEN Inc., Valencia, CA) and were sequenced to confirm sequence identity (Sangon Corp., Shanghai, China).

The HTR8/Svneo cells were seeded in 60 mm dishes at 5.0×10^5 cells/dish and grown overnight to 50% confluence prior to transfection. All plasmids were transfected with Effectene® Transfection Reagent (QIAGEN) following the manufacturer's instructions. The transfection experiment was performed with 1.0 μg siRNA expression plasmids for 48 h. To select hygromycin resistant cells, 200 μg/ml hygromycin B (Sangon) was applied for 3–4 days. Resistant cells were cultured and passaged until sample collection.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA from 2.0×10^6 cells was extracted using Trizol reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions. The first-strand cDNA was synthesized with Superscript II reverse transcriptase (Gibco) and oligo dT from 2 μg of total RNA. As internal controls for RT, samples without RNA or without reverse-transcriptase were prepared in parallel. All PCR reactions were conducted in the exponential range of amplification for each set of primers. Specific PCR primer pairs and the different parameters of amplification are summarized in Table 1, and the same parameters for all PCR amplifications were as follows: denaturation, 45 sec at 94°C; extension, 45 sec at 72°C. All PCR reactions were performed in a final volume of 25 μl containing 2 μl of the first strand cDNA, 200 μM dNTPs, 2 mM $MgCl_2$, 1U *Taq* polymerase (TaKaRa Corp., Dalian, China), and 10 pmol of each primer. As negative controls for PCR, samples without first-strand cDNA or without Taq enzyme were used. Amplification of GAPDH gene transcripts was used to confirm RNA integrity and efficiency. All control reactions yielded negative results (data not shown).

TABLE 1. The sequences for specific PCR primer pairs and the different parameters for PCR amplification

Gene		Primer pairs sequence (5′ → 3′)	Annealing	Cycles	Product (bp)
LMP2	sense	GTTGTGATGGGTTCTGATTC	59°C/45 s	28	349
	antisense	CACCAATGGCAAAAGGCT			
IκBα	sense	GCTGCCCTATGATGAC	58°C/45 s	27	422
	antisense	CACTGAACGCTTAACACT			
p50	sense	GCTGGCTGAAGATGTGAA	59°C/45 s	28	379
	antisense	GAGTTTGCGGAAGGATGT			
p65	sense	CAGCCCTATCCCTTTAC	57°C/45 s	28	277
	antisense	CCTCTGACAGCGTTCC			
MMP-2	sense	GAAGAGCGTGAAGTTTGG	59°C/45 s	33	291
	antisense	TCTGAGGGTTGGTGGG			
MMP-9	sense	GGGACGGCAATGCTGATG	60°C/45 s	35	320
	antisense	CCACTTCTTGTCGCTGTC			
GAPDH	sense	AGCCACATCGCTCAGACAC	58°C/45 s	23	315
	antisense	TGGACTCCACGACGTACTC			

Gelatin zymography

The cell culture media were harvested after incubating with media without fetal bovine serum for 24 h. The media were mixed with four-strength sample buffer (8% SDS (w:v), 0.04% bromophenol blue (w:v), 0.25 M Tris), incubated at 37°C for 30 min, and 10 μg total protein was subjected to SDS–polyacrylamide electrophoresis (SDS–PAGE) in 10% polyacrylamide gels containing 0.5 mg/ml gelatin (Difco Laboratories, Detroit, MI). After electrophoresis, the gels were washed twice in 2.5% Triton X-100 and 50 mM Tris-HCl (pH 7.5) at room temperature to remove SDS, and then incubated with calcium assay buffer (50 mM Tris, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100, pH 7.5) for 24 h at 37°C. The gels were stained with Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid for 1 h at room temperature and destained with 10% acetic acid. MMPs exhibiting gelatinolytic activity appeared as clear bands. Estimation of molecular weights was possible owing to the concurrent electrophoresis of different molecular weight protein markers.

Preparation of cytosolic and nuclear extracts

Cells (2.0×10^6) were harvested and extracted with the Nuclear Extract Kit (Active Motif North America, Carlsbad, CA) according to the manufacturer's protocols. Briefly, the cells were washed with ice-cold PBS/phosphatase inhibitors ($1 \times$ PBS, 5% (v/v) phosphatase inhibitor) and centrifuged. The cell pellets were resuspended and incubated in $1 \times$ hypotonic buffer on ice and then treated with detergent reagent. After centrifugation, the supernatant was collected to detect cytosolic proteins, and the pellet was resuspended with the complete lysis buffer to extract nuclear proteins. The supernatant from subsequent centrifugation was harvested and saved for nuclear protein analysis.

Western blotting

Whole cell proteins were extracted with Trizol Reagent after total RNA extraction according to the manufacturer's instructions. Quantification of proteins was determined by UV spectrophometer (Bechman DU530, Fullerton, CA), and 20 μg of proteins were subjected to SDS–PAGE on 12% or 15% (w/v) gels according to the molecular weight of protein of interest. Separated proteins were transferred electrophoretically onto a Pure Nitrocellulose Blotting membrane (Pall Corporation, Pensacola, FL), and then incubated with blocking buffer (3% BSA (v/v) in TBST) for 1 h at room temperature. The membrane was subsequently incubated with appropriate primary antibodies overnight at 4°C (LMP2: 0.2 μg/ml, Abcam Ltd., Cambridge, UK; other antibodies: 0.8 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), washed three times with TBST for 15 min each time at room temperature, incubated for 1 h at room temperature with TBST containing alkaline phosphatase-conjugated secondary antibodies, washed three times with TBST and once with TBS, and subjected to the alkaline phosphatase color reaction by standard methods. The detection for actin and Lamin A/C protein was performed as internal controls for cytosolic and nuclear proteins, respectively.

Immunofluorescence microscopy

Cells at 2.0×10^5 were seeded to coverslips and harvested at 60% confluence. The slides were washed in PBS twice and fixed with pre-chilled methanol/acetone (1:1) at −20°C for 30 min. After washed in TBST, the cells were blocked with washing buffer containing 3% BSA (v/v) for 1 h at room temperature. The anti-LMP2 antibody (Abcam Ltd.) was used at 20 μg/ml (diluted in blocking buffer) for 1 h at 37°C. After three washes with TBST, cells were incubated with 20 μg/ml green FITC-labeled secondary antibody for 1 h at 37°C, then washed three times with TBST and once with TBS. The coverslips were then rapidly rinsed in PBS and water before being mounted with DABCO reagent, and positive signals were recorded by fluorescence microscopy (Leica, Deerfield, IL).

Statistics

Quantification of the bands from RT-PCR, Western blotting, and gelatin zymography were determined by MetaView Image Analyzing System (Version 4.50; Universal Imaging Corp., Downingtown, PA). Relative levels of mRNA and protein normalized to the corresponding internal controls were calculated, and activities of MMP-2 and MMP-9 detected by zymography were quantified by densitometric analysis of the intensities of the bands. Statistical analysis was performed using the Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS Inc., Chicago, IL). One-way ANOVA was used to analyze statistical differences. $P < 0.05$ was considered to be statistically significant.

RESULTS

Inhibition of LMP2 by vector-based RNAi in the HTR8/Svneo cell line

It has been reported that vector-based siRNA can successfully knock down specific gene expression for an extended period in mammalian cells (Brummelkamp et al., 2002; Sui et al., 2002). To explore the regulatory roles of LMP2 in the HTR8/Svneo cell line, a vector-based LMP2 siRNA plasmid was transfected into this cell line. The results showed that LMP2 mRNA was reduced by 70%, and its protein level decreased by 65%, in the group transfected with the LMP2 siRNA plasmid compared to the normal cell group ($P < 0.05$). The expression levels of LMP2 mRNA and protein in the group transfected with the negative plasmid were consistent with those in the normal cell group ($P > 0.05$) (Fig. 1A–C). Internal controls used in this study (GAPDH for mRNA and actin for protein) showed no differences among the three cell groups (Fig. 1A–C).

As reported previously (Brooks et al., 2000), the LMP2 protein is strongly enriched around the endoplasmic reticulum in normal HTR8/Svneo cells and in the cells transfected with negative plasmid, and weakly expressed in the nucleus and throughout the cytosol (Fig. 1D,H, and N). However, the cells transfected with LMP2 siRNA plasmid showed only faint signals for LMP2 protein in the cytosol and nucleus (Fig. 1D,L).

Inhibition of LMP2 reduces MMP-2 and MMP-9 mRNA expression and their activities

To further understand the effect of LMP2 inhibition on MMP-2 and MMP-9, we examined the expression of MMP-2 and MMP-9 mRNA and their activities. Using RT-PCR, the expression of MMP-2 and MMP-9 mRNA was found to be markedly reduced in the LMP2-inhibited cells compared with the two control groups of cells ($P < 0.05$) (Fig. 2A,B). With zymography, gelatinolytic activities were detected as a major band of 92 kDa produced by the pro-form of MMP-9 and a minor band of 72 kDa corresponding to the pro-form of MMP-2 in the HTR8/Svneo cell line (Fig. 2C). The active forms of MMP-2 and MMP-9 were not observed. The activities of pro-MMP-2 and pro-MMP-9 were markedly inhibited in the LMP2-inhibited cells ($P < 0.05$) (Fig. 2C,D).

Effect of LMP2 inhibition on the expression of IκBα

IκBα is an inhibitory protein that masks the p65 nuclear-localization signal (NLS), which can modulate the nuclear import of active p50–p65 complex. UPP plays important roles in IκBα degradation (Chen et al., 1995; Lin et al., 1998; Sears et al., 1998). To identify the possible effect of LMP2 on IκBα, its expression was analyzed in the three cell groups. In the LMP2-inhibited HTR8/Svneo cells, IκBα mRNA was markedly reduced ($P < 0.05$) (Fig. 3A,B), but phosphorylated IκBα (p-IκBα) increased while non-phosphorylated IκBα (non-p-IκBα)

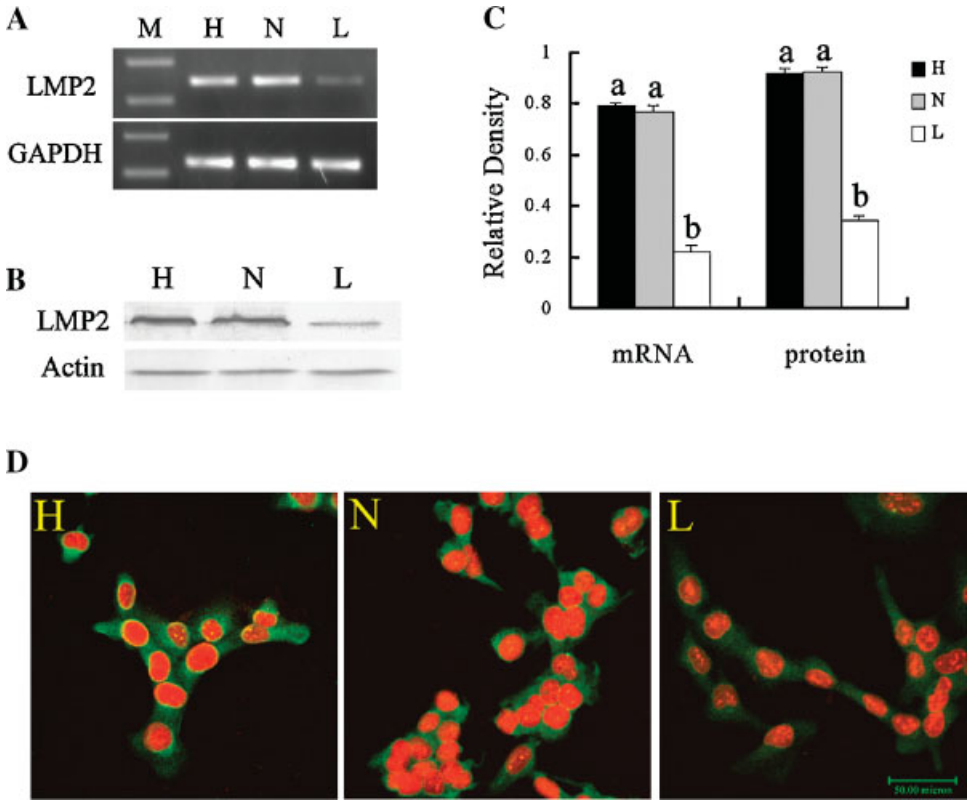

Fig. 1. Vector-based RNAi knocks down LMP2 expression in the human invasive extravillous trophoblast cell line (HTR8/Svneo). **A**: RT-PCR detection of LMP2 mRNA expression in the three cell groups (H, N, L). M, DNA marker; H, normal HTR8/Svneo cells; N, HTR8/Svneo cells transfected with the negative RNAi plasmids; L, HTR8/Svneo cells transfected with LMP2 RNAi plasmids. The same abbreviations are used in the following figures. The GAPDH transcript was amplified as an internal control. **B**: Western blotting analysis of LMP2 protein in the three cell groups (H, N, L). Actin protein was used as an internal control. **C**: Statistical analysis of LMP2 mRNA and protein in the three cell groups (H, N, L). The relative level for LMP2 was determined as the ratio of LMP2 mRNA and protein to its corresponding internal control as measured by densitometry. Bars with different letters among the same class are significantly different ($P < 0.05$). **D**: Cellular location of LMP2 protein in the three cell groups (H, N, L). Bar = 50.00 μm.

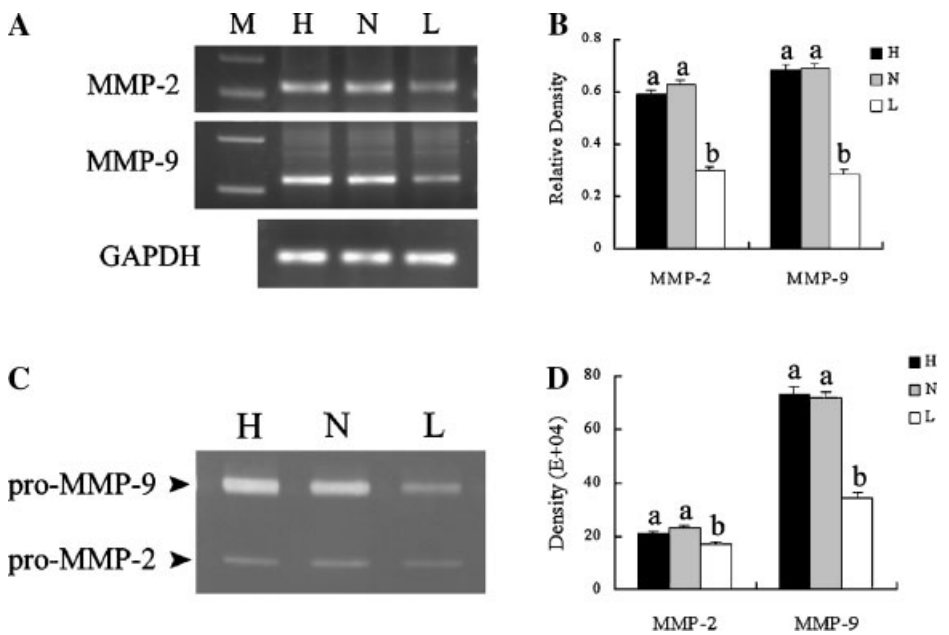

Fig. 2. The effect of LMP2 inhibition on MMP-2 and MMP-9 mRNA expression and their activities. **A**: RT-PCR analysis of the expression of MMP-2 and MMP-9 mRNA in the three cell groups. **B**: Statistical analysis of MMP-2 and MMP-9 mRNA in the three cell groups. The ratio of MMP-2 and MMP-9 mRNA to GAPDH mRNA was measured by densitometry. Bars with different letters among the same class are significantly different ($P < 0.05$). **C**: Representative gelatin zymogram of MMP-2 and MMP-9 in the three cell groups. The molecular weights of the two bands are 92 kDa for pro-MMP-9 and 72 kDa for pro-MMP-2. **D**: Activities of MMP-2 and MMP-9 detected by gelatin zymogram were quantified by computer-aided densitometry. Bars with different letters among the same class are significantly different ($P < 0.05$).

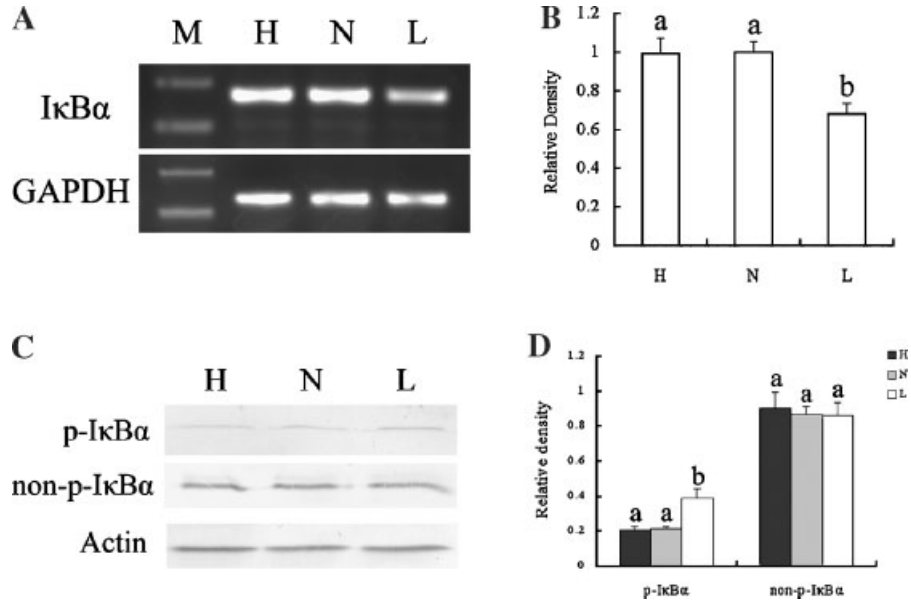

Fig. 3. The effect of LMP2 inhibition on the expression of IκBα in the HTR8/Svneo cells. **A**: Representative RT-PCR products of IκBα and GAPDH (internal control) in the three cell groups. **B**: Statistical analysis of IκBα mRNA level in the three cell groups. The ratio of IκBα mRNA to GAPDH mRNA was measured by densitometry. Bars with different letters are significantly different ($P < 0.05$). **C**: Immunoblot analysis of IκBα proteins (p-IκBα and non-p-IκBα, respectively) and actin (used as the internal control) in the three cell groups. **D**: Statistical analysis of IκBα proteins in the three cell groups. The ratio of IκBα proteins to actin protein was measured by densitometry. Bars with different letters among the same class are significantly different ($P < 0.05$).

protein level remained normal relative to that in the two control cell groups (Fig. 3C,D).

Effect of LMP2 inhibition on expression of NF-κB subunits p50 and p65

Since p50 and p65 are two important functional subunits for NF-κB heterodimers (Hayden and Ghosh, 2004), changes of p50 and p65 expression in the LMP2-inhibited HTR8/Svneo cells were also studied. The expression levels of p50 and p65 mRNA did not differ markedly ($P > 0.05$) (Fig. 4A,C, and D), but when the basal expression of p50 and p65 proteins in the cytosolic and nuclear extracts was examined by Western blotting analysis, the expression of p50 proteins in the cytosolic and nuclear extracts, and of p65 protein in the nuclear extract was significantly reduced ($P < 0.05$). The expression of p65 protein in the cytosolic extract was not affected in the LMP2-inhibited HTR8/Svneo cells

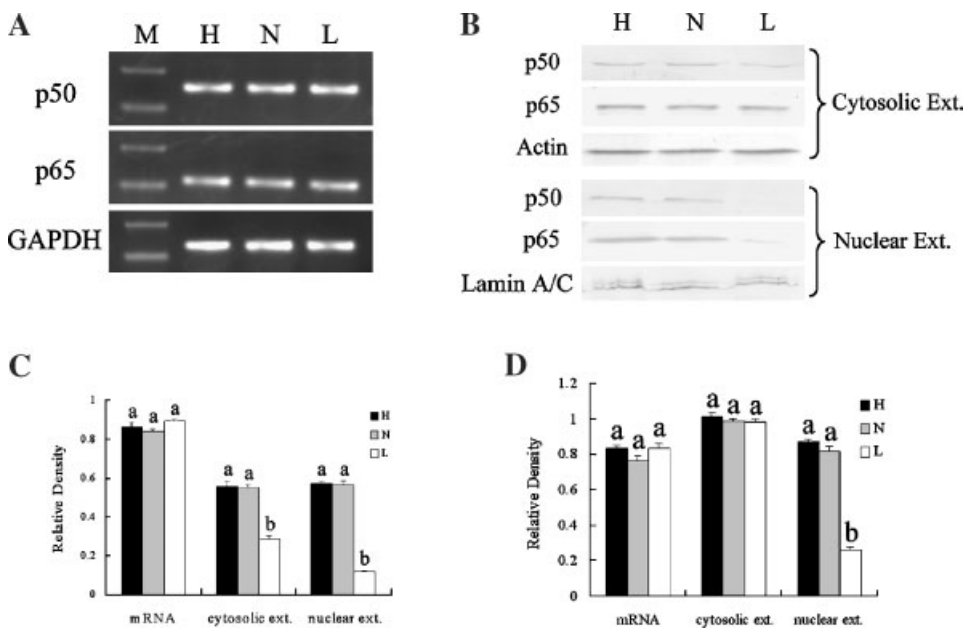

Fig. 4. The effect of LMP2 inhibition on the expression of NF-κ subunits p50 and p65 in the HTR8/Svneo cells. **A**: Representative RT-PCR products of p50, p65, and GAPDH (internal control) in the three cell groups. **B**: Immnuoblot detection of p50 (cytosolic and nuclear extracts), p65 (cytosolic and nuclear extracts), actin (used as the internal control for cytosolic protein), and Lamin A/C (used as an internal control for the nuclear protein). **C–D**: Statistical analysis of mRNA and protein for p50 (C) and p65 (D) in the three cell groups. The relative levels for p50 and p65 were determined by the ratio of p50 and p65 mRNA and protein to their corresponding internal control measured by densitometry. Bars with different letters among the same class are significantly different ($P < 0.05$).

($P > 0.05$) (Fig. 4B–D). Cells transfected with the negative plasmid showed no expression difference NF-κB subunits compared with the normal HTR8/Svneo cells ($P > 0.05$) (Fig. 4A–D).

SN50 inhibits MMP-2 and MMP-9 expression and their activities

SN50 is known to inhibit subcellular trafficing of NF-κB complexes from the cytoplasm to the nucleus (Lin et al., 1995), so to confirm the effect of NF-κB on MMP-2 and MMP-9 expression, we utilized this synthetic peptide inhibitor. At 50 μg/ml of SN50, the expression of MMP-2 and MMP-9 mRNA was significantly decreased, and the activities of MMP-2 and MMP-9 were also markedly reduced ($P < 0.05$) (Fig. 5A–D). The inhibitory effect on MMP-9 was more significant than that on MMP-2 at the mRNA and protein levels (Fig. 5B,D). Cells treated with an equivalent volume of DMSO (the solvent for SN50) showed no alteration in MMP-2 and MMP-9 expression or their activities ($P > 0.05$) (Fig. 5C,D).

DISCUSSION

Trophoblast invasion and extensive degradation and remodeling of ECM are key events for successful implantation (Carson et al., 2000). Some proteinases produced by trophoblast and proteolytic pathways, such as MMP-2, MMP-9, and UPP, are involved in these events (Cross et al., 1994; Polette et al., 1994; Xu et al., 2002). Our previous studies demonstrated that MMP-2, MMP-9, and the UPP play important roles in embryo implantation, and that UPP blockage markedly reduces the expression and activity of MMP-2 and MMP-9 (Wang et al., 2004a). Meanwhile, the NF-κB pathway has been confirmed to play a role in the regulation of MMP-2 and MMP-9 expression (Han et al., 2001; Hozumi et al., 2001). LMP2, strongly expressed in the trophoblastic cells, is a key β-type subunit for catalytic activity in the 20S proteasome (Kuchelkorn et al., 1995; Wang et al., 2004b). In LMP2 knockout mice, the proteasome function was impaired and the incidence of uterine tumor increased (Hayashi and Faustman, 2002). However, the complex interactions between LMP2, NF-κB, MMP-2, and MMP-9 that contribute to successful implantation are not fully understood. This study addresses these interactions and the possible regulation of the expression of MMP-2 and MMP-9 by LMP2 via the NF-κB pathway.

RNAi is a post-transcriptional gene silencing process in which double-stranded RNA (dsRNA) causes the degradation of homologous mRNA sequences (Hannon, 2002). Vector-based RNAi has become a powerful genetic tool to stably suppress gene expression in mammalian cell lines (Brummelkamp et al., 2002; Denti et al., 2004). In this study, we stably suppressed the expression of LMP2 mRNA and protein in the HTR8/Svneo cell line using vector-based RNAi technology, which assisted our investigation into the regulatory role of LMP2 in the UPP.

As an extension of our previous studies showing that inhibition of UPP by lactacystin decreased expression and activities of MMP-2 and MMP-9 in vitro (Wang et al., 2004a), we further demonstrated that the expression and activities of MMP-2 and MMP-9 were markedly suppressed in the LMP2-inhibited HTR8/Svneo cells. Although Ikebe et al. (1998) reported that the proteasome inhibitor, lactacystin, could prevent TNF-alpha from enhancing MMP-9 production and inhibit NF-κB activation, the precise mechanism for this regulatory process remains to be elucidated.

The UPP plays an essential role in two distinct steps for activation of the NF-κB pathway including degrada-

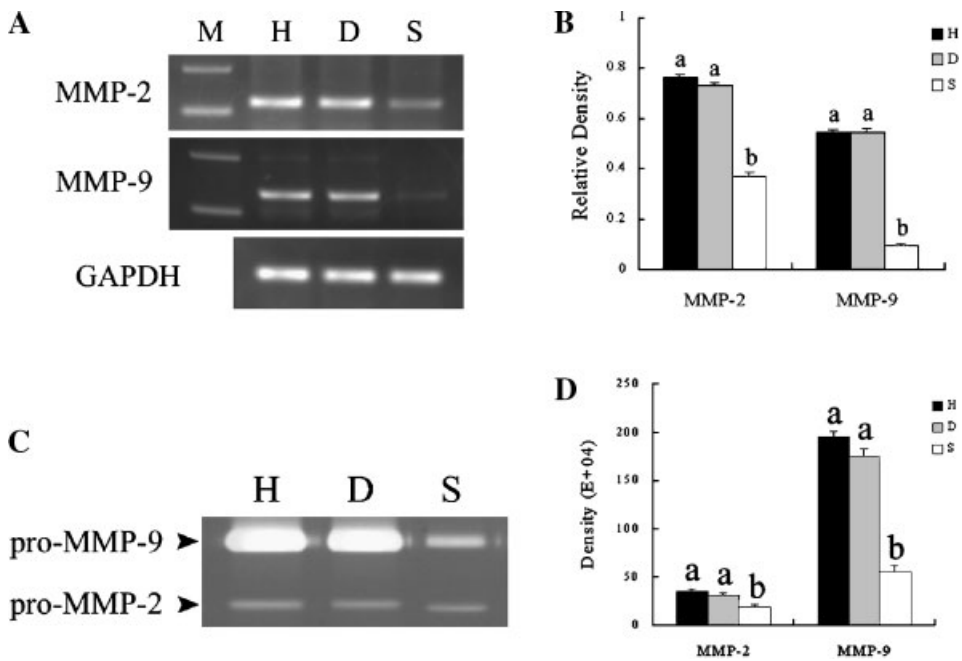

Fig. 5. The inhibitory effect of NF-κB inhibitor SN50 on mRNA expression and the activities of MMP-2 and MMP-9. **A**: Agarose-gel electrophoresis of MMP-2 and MMP-9 in the three cell groups (H, D, S). M, DNA marker; H, normal HTR8/Svneo cells; D, cells treated with DMSO; S, cells treated with SN50. **B**: Statistical analysis of MMP-2 and MMP-9 mRNA in the three cell groups. The ratio of MMP-2 and MMP-9 mRNA to GAPDH mRNA was measured by densitometry. Bars with different letters among the same class are significantly different ($P < 0.05$). **C**: Detection of MMP-2 and MMP-9 activities in the three cell groups by gelatin zymography. The molecular weights of the two bands are 92 kDa for pro-MMP-9 and 72 kDa for pro-MMP-2. **D**: Statistical analysis of MMP-2 and MMP-9 activities in the three groups of cells. The density of bands was quantified by computer-aided densitometry. Bars with different letters among the same class are significantly different ($P < 0.05$).

tion of phosphorylated IκBα and generation of NF-κB subunit p50 (Chen et al., 1995; Lin et al., 1998; Sears et al., 1998). Our results showed that LMP2 inhibition didn't affect the expression of non-phosphorylated IκBα, however, the degradation of phosphorylated IκBα by UPP was impaired because overexpression of phosphorylated IκBα was found in the LMP2-inhibited HTR8/Svneo cells. A similar result was confirmed in the LMP2 knockout mice (Hayashi and Faustman, 2002). Although the expression of p65 in the cytosolic extract appears normal in the LMP2-inhibited HTR8/Svneo cells, expression of p50 in the cytoplasm and nucleus, as well as of p65 in the nucleus, were markedly suppressed. These results indicate that the proteasome subunit LMP2 is required for the degradation of phosphorylated IκBα, for the generation of p50, and for blocking the translocation of NF-κB heterodimers into the nucleus. Our data provide further evidence for the mechanistic view that regulation of the NF-κB pathway by UPP depends mainly on the LMP2 subunit in the 20S proteasome core (Hayashi and Faustman, 2000). Once in the nucleus, NF-κB will induce the expression of multiple host genes, including the IκBα gene (Chen and Greene, 2004). Thus, the decrease of IκBα mRNA expression in the LMP2-inhibited HTR8/Svneo cells is probably triggered by the blockage of NF-κB passage into the nucleus.

Previous studies have demonstrated that NF-κB is involved in regulating the expression and activity of MMP-2 and MMP-9, but the results from differing cell lines are varied (Woo et al., 2003; Xie et al., 2004). SN50 is an important NF-κB inhibitor that is known to inhibit the subcellular translocation of NF-κB from the cytoplasm to the nucleus (Lin et al., 1995). Our data showed that SN50 significantly decreased MMP-2 and MMP-9 mRNA expression and their activities, and that the inhibitory effect upon MMP-9 is stronger than that on MMP-2. Because the proximal stimulatory region of the MMP-9 promoter has a functional NF-κB binding site (Huhtala et al., 1991), inhibition of the NF-κB pathway is likely responsible for suppressing MMP-9 mRNA expression and its activity. Although no consensus NF-κB binding site has been identified in the MMP-2 promoter, activation of MMP-2 is regulated by membrane-type 1 MMP (MT1-MMP), which does have an NF-κB binding site in its promoter (Strongin et al., 1995). Therefore, it is possible that NF-κB regulates MMP-2 activity indirectly via MT1-MMP, and that NF-κB directly modulates MMP-2 expression and activity via its interaction with other transcription factors that regulate MMP-2 expression (Hernandez-Barrantes et al., 2000; Han et al., 2001). These possibilities are supported by our results that NF-κB has a stronger inhibitory effect on MMP-9 than that of MMP-2.

The important and interesting finding of this study is that the regulatory effect of LMP2 inhibition on MMP-2 and MMP-9 expression and activities is fully consistent with the results of NF-κB blockage by SN50. Therefore, it is reasonable to draw the conclusion that the proteasome subunit LMP2 is essential for regulating the expression and activities of MMP-2 and MMP-9 in the HTR8/Svneo cell line, and that the regulatory pathway is mediated, at least in part, through the NF-κB pathway.

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