NF-KAPPA B ACTIVATION REPRESSIONS TNFALPHA-INDUCED AUTOPHAGY.

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SUMMARY

Activation of NF-kappaB and autophagy are two processes involved in the regulation of cell death, but the possible cross-talk between these two signaling pathways is largely unknown. Here we show that NF-kappaB activation mediates repression of autophagy in TNFalpha-treated Ewing sarcoma cells. This repression is associated with an NF-kappaB-dependent activation of the autophagy inhibitor mTOR. In contrast, in cells lacking NF-kappaB activation, TNFalpha treatment upregulates the expression of the autophagy-promoting protein Beclin 1, and subsequently induces the accumulation of autophagic vacuoles. Both of these responses are dependent on reactive oxygen species (ROS) production and can be mimicked in NF-kappaB-competent cells by the addition of H₂O₂. Small interfering RNA-mediated knock down of beclin 1 and atg7 expression, two autophagy-related genes, reduced TNFalpha- and ROS-induced apoptosis in cells lacking NF-kappaB activation and in NF-kappaB-competent cells, respectively. These findings demonstrate that autophagy may amplify apoptosis when associated with a death signaling pathway. They are also evidence that inhibition of autophagy is a novel mechanism of the anti-apoptotic function of NF-kappaB.
activation. We suggest that stimulation of autophagy may be a potential way bypassing the resistance of cancer cells to anticancer agents that activate NF-kappaB.

INTRODUCTION

Acquisition of drug resistance in cancer cells is the major cause of the inefficacy of cancer therapy. Activation of the NF-κB transcription factor is one of the signaling pathways that contributes to the resistance of cancer cells to radio- and chemotherapies (1-4). Indeed, inactivation of NF-κB sensitizes numerous cancer cell lines to the cytotoxic effect of anti-cancer treatments (1-4).

NF-κB is an ubiquitously expressed family of Rel-related transcription factors (5). Typically, in unstimulated cells, NF-κB is sequestered in the cytoplasm by binding to inhibitory κB proteins (IκB). In response to a variety of stimuli such as inflammatory cytokines, oncogenes, and viruses, the proteasome-dependent degradation of IκB allows the translocation of NF-κB to the nucleus and its binding to the promoter region of target genes involved in the control of different cellular responses including apoptosis (6-8). In many cancer cells, the constitutive activation of NF-κB activity lowers cell sensitivity to apoptosis and consequently favors neoplastic cell survival (9). Several anti-tumor drugs appear to enhance NF-κB activity which renders them less effective (10).

Recently, evidence has emerged that autophagy is another mechanism involved in the control of death in cancer cells (11,12). Macroautophagy (hereafter referred to as autophagy) is a vacuolar lysosomal degradation pathway for organelles and cytoplasmic macromolecules (13,14). Genetic studies of autophagy in the yeast Saccharomyces cerevisiae have led to the identification of a family of genes named ATG involved in the control of autophagy (15-17). The formation of autophagosomes requires two conjugation systems (Atg5-Atg12 and Atg8 lipidation) (18) and a class III phosphatidylinositol 3-kinase (19). This kinase interacts with the tumor suppressor protein Beclin 1, the mammalian orthologue of the yeast Atg 6 (20).

The relation between autophagy and cell death is complex since autophagy can be involved in either cell death or survival depending on the cellular context (12,21,22). The survival function of autophagy has been demonstrated under different physiological situations, such as interruption of maternal nutrient supply in newborn mice (23) or cell deprivation of growth factors and of nutrients (24,25). Autophagy is also implicated in the cell death process during development (26), and in response to several cytotoxic stimuli (reviewed in Ref. (27)). In some situations, both apoptosis and autophagy can occur concomitantly in the same cells, suggesting the involvement of common regulatory mechanisms (28). In fact, several members of both extrinsic (29,30) and intrinsic (31) apoptotic pathways can promote activation of autophagy. In particular, death receptor ligands, such as TNFα (30) and Trail (29), stimulate autophagy in T-lymphoblastic cells.
and in a model of lumen formation in mammary acini, respectively. In addition, some participants in apoptosis signaling, such as the receptor interacting proteins RIP (32) and FADD (33), and the kinase JNK (32), have recently been implicated in autophagy. The precise network that controls the cross-talk between apoptosis and autophagy remains to be elucidated. In particular, it has not yet been investigated whether NF-κB, one of the major regulators of apoptosis, also controls autophagy.

Here, we examined whether NF-κB activation modulates the autophagic capacity of cells. We compared the autophagic capacity of NF-κB competent cells with that of cells carrying a repressor of NF-κB activation after treatment with TNFα. Whereas TNFα induced autophagy in cells lacking NF-κB activity, it did not activate this process in NF-κB competent cells. We also demonstrate that in the absence of NF-κB activation, TNFα-induced autophagy is dependent on ROS production and participates in the TNFα-induced apoptotic signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents

Hoechst 33258 dye, benzon nuclease, 3-methyladenine, monodansylcadaverine (MDC), hydrogen peroxide, butylated hydroxyanisole (BHA) and N-butyl-α-phenyl-nitrone (BNP) were from Sigma (St Louis, MO). \([^{14}C] \text{L-valine} (5.47 \text{ GBq/mmol})\) was from Perkin Elmer Life Science (Wellesley, MA USA). Z-VAD-fmk and recombinant human TNFα (TNFα was used in cells at a concentration of 2000 Unit/ml) were from R&D Systems, Inc. (Minneapolis, MN, USA). Antibodies against the following proteins were used: Atg5 (Santa Cruz Biotechnology, CA, USA), Atg7 (kindly provided by Dr. W. A. Jr Dunn, University of Florida College of Medicine, Gainesville, FL, USA), Beclin 1 and actin (BD Biosciences, Heidelberg, Germany), phospho-4-EBP1, 4-EBP1, phospho-p70 S6kinase, p70 S6 kinase, Bcl-2 (Cell Signaling Technology, Inc. MA, USA) LC3 (kindly provided by Dr. Eiki Kominami, Jutendo University, Tokyo, Japan and Dr. Tamotsu Yoshimori, National Institute of Genetics, Mishima, Japan), PARP (Alexis, Illkirch, France).

Cells

EW7 cells transfected with an empty pcDNA vector (EW7PC cells) or with the IkBα(A32/36)-encoding vector (EW7MAD cells) were previously described (34). The generation of promyelocytic leukemia cells (NB4) expressing either the Migr-eGFP vector (NB4/GFP cells) or the IkBα(A32/36)-encoding Migr-eGFP (NB4/GFP-MAD) was previously described (35). All of these cells were grown at 37°C in 5% CO₂ RPMI medium supplemented with 2 mM L-glutamine and 10% decomplemented fetal calf serum. Human breast cancer MCF7 cell line (from ATCC, Parklawn Drive, Rockville, Canada) were grown at 37°C in 10% CO₂ in DMEM supplemented with 10% decomplemented fetal calf serum.
**MDC staining**

MDC was used to evaluate the abundance of autophagic vacuoles in cells as previously reported (36). A 10 mM stock solution of MDC was prepared in DMSO. Following treatment, cells were stained with MDC at a final concentration of 10 µM, for 10 minutes at 37°C, and then collected and fixed using 3% paraformaldehyde solution in PBS for 30 minutes. Cells were washed and then examined by fluorescence microscopy (Zeiss Axioplan microscope). For each condition, the percentage of cells with characteristic MDC staining dots indicative of autophagy was assessed.

**Analysis of degradation of long-lived proteins**

Ewing sarcoma-derived cell lines were incubated with 0.2 µCi/ml of [14C] L-valine in complete medium (RPMI medium supplemented with 2 mM L-glutamine and 10% FCS) for 24 h at 37°C (37). At the end of the radiolabeling period, unincorporated radioisotopes were removed by washing the cells three times with phosphate buffer saline (pH 7.4). Cells were then incubated in complete medium supplemented with 10 mM unlabeled valine for 1 h (pre-chase period). After this time, the medium was replaced by either nutrient-free medium (Hanks balanced Salt solution plus 0.1% bovine serum albumin) or complete medium plus 10 mM unlabeled valine in the presence and absence of 10 mM 3-MA and TNFα for 4 to 8 h incubation (chase period). Radiolabeled proteins from the medium and adherent cells were precipitated in trichloroacetic acid (TCA) at the final concentration of 10% (v/v), separated from soluble radioactivity by centrifugation at 600 g for 20 min, and dissolved in 1 ml of 0.2 N NaOH. The rate of protein degradation was calculated by determining the ratio of acid-soluble radioactivity recovered from cells and medium to the ratio of radioactivity in TCA-precipitated proteins obtained from cells and medium.

**Electron microscopy**

Cells were fixed for 1 h at 4°C in 1.6% glutaraldehyde in 0.1 M Sörensen phosphate buffer (pH 7.3), washed and fixed again in aqueous 2% osmium tetroxide, dehydrated in ethanol, embedded in Epon, and processed for electron microscopy with a Zeiss EM 902 transmission electron microscope at 80 kv, in ultrathin sections stained with uranyl acetate and lead citrate.

**Detection of apoptosis**

Apoptotic cell death was determined by quantification of apoptotic nuclei (i.e. fragmentation and condensation of nuclei) following Hoechst 33258 staining. A total of 500 nuclei were counted for each sample. Apoptosis was also evaluated by determination of caspase 3 activity which was assessed by the appearance of PARP1 cleaved product revealed by Western blotting analysis. DNA fragmentation was quantified using a cell death detection Elisa plus kit (Roche diagnostics GmBH, Mannheim, Germany), which was used according to the manufacturer’s instructions.
**Isolation of RNA and real-time quantitative RT-PCR.**

Total RNA was extracted with the RNeasy Mini kit (Qiagen, Courtaboeuf, France). First-strand cDNA was generated by reverse-transcription of 2 μg of total RNA using random primers and Superscript™ III reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s instructions, in a total reaction volume of 20 μl. The sequences of forward and reverse oligonucleotide primers, specific to the chosen candidate and housekeeping genes, were designed using Primer3 software: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The primers are for Beclin 1 and β-actin [forward, 5'GGCTGAGAGACTGGATCAGG 3’ and reverse, 5'CTGCGTCTGGGCATAACG 3’; GenBank NM003766, nucleotide 787-913] and [forward, 5'TCACCCACACTGTGCCCATCTACGA3’ and reverse, 5'CAGCGGAACCGCTCATTGCCAATGG3’; GenBank: BC016045, nucleotide 555-845], respectively.

Real-time quantitative PCR was performed in a LightCycler® (Roche Diagnostics, Meylan, France) thermal cycler. A 80 fold dilution of each cDNA was amplified in a 10-μl volume, using the Fast Start DNA MasterPLUS SYBR Green I master mix (Roche Diagnostics), with 500 nM final concentrations of each primer. The amplification specificity was checked by melting curve analysis and gel agarose electrophoresis of PCR products. Threshold cycle Ct, which correlates inversely with the target mRNA levels, were calculated using the second derivative maximum algorithm provided by the Lightcycler software. For each cDNA, the Beclin 1 mRNA levels were normalised to β-actin mRNA levels. Results are expressed as ratio of normalised Beclin1 mRNA level of treated cells to those of untreated cells.

**Transfection and RNA interference**

Small interfering RNAs (siRNAs) against beclin 1, atg7, p65 and control siRNA were synthesized by Eurogentec (Seraing, Belgium). The siRNA sequence against beclin-1, atg7, p65 were previously described respectively in (25,38,39). Cells cultured in 6-well plates were transfected with siRNA at 200 nM final concentration by using oligofectamine reagent (Invitrogen, Cergy-Pontoise, France). Cells were then incubated for 8 h at 37°C prior to addition of 5% fetal calf serum and then left for another 48 h-92 h. At the end of these treatments, cells were harvested and subjected to Western blotting analysis or apoptosis assays.

Transient transfections with GFP-LC3 plasmids (kindly provided by Dr. Tamotsu Yoshimori, National Institute of Genetics, Mishima, Japan) were carried out by using lipofectamine 2000 transfection reagent according to the manufacturer’s instructions. Since the level of transfection in Ewing sarcoma cells only reach 15% of the total amount of cells, 2x10⁶ cells were transfected for each assay and then subjected to fluorescence microscopy analysis. For each
condition, at least 150 GFP-LC3 transfected cells (GFP-expressing cells) were observed and the percentage of transfected cells presenting punctuate GFP-LC3 staining, which is indicative of autophagy structures, was determined.

Western blot analysis

Cellular extracts were prepared in 10 mM Tris pH 7.4, 1% SDS, 1 mM sodium vanadate, treated with benzon nuclease for 5 min at room temperature and boiled for 3 min. Fractions (30 µg) of cellular extract proteins were subjected to SDS-polyacrylamide gel electrophoresis using a Tris/glycine buffer system based on the method of Laemmli. After electrophoresis, proteins were transferred to a Protean nitrocellulose transfer membrane (Amersham Biosciences, Orsay, France). Protein loading was assessed by Ponceau staining of membranes. Blots were then incubated with primary antibodies using the manufacturer’s protocol followed by the appropriate horseradish peroxidase-conjugated secondary antibody. Immunostained proteins were visualized on X-ray film using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). The experiments were repeated at least three times and representative autoradiograms are shown.

RESULTS

Inhibition of NF-κB activation triggers autophagy in TNFα-treated Ewing sarcoma cells.

To evaluate the relation between NF-κB activation status and autophagy, we compared the autophagic activity after TNFα treatment in cells lacking NF-κB activity (EW7MAD1) and NF-κB-competent (EW7PC) Ewing sarcoma cells. The formation of autophagic vacuoles was first assessed by staining with monodansylcadaverine (MDC), which accumulates in acidic cell compartments enriched in lipids (36). This leads to a punctuate staining pattern when autophagy is stimulated (36). As shown in figure 1A, both control cells (EW7PC) and cells expressing the repressor of NF-κB activation (EW7MAD1) presented diffuse staining in the absence of TNFα. TNFα treatment of cells lacking NF-κB activity resulted in the appearance of punctuate structures 4 h after treatment. In addition, the number and size of these punctuate structures were significantly increased following 8 h of TNFα treatment, suggesting that TNFα induces the accumulation of autophagic vacuoles in these cells. Conversely, the accumulation of autophagic vacuoles by TNFα is impaired in NF-κB-competent cells (EW7PC) as revealed by the paucity of punctuate structures observed in these cells. To verify that EW7PC cells are not defective in stimulation of autophagic activity, MDC staining was also performed following incubation of these cells in nutrient-free medium, a condition known to stimulate autophagy (14). Under these conditions, EW7PC cells as well as EW7MAD1 cells were both able to induce autophagy (figure 1A). The redistribution of LC3 from diffuse cytosolic
staining to punctuate staining is a reliable marker of autophagosome formation (40). This can be examined either by transfection of cells using GFP-LC3 expression plasmids which result in the formation of punctuate fluorescence structures in conditions of autophagy stimulation, or by examining the appearance of the phosphatidylethanolamine (PE) conjugated form of LC3, (LC3-II) which is associated with autophagosomal membranes. After cell transfection with GFP-LC3, TNFα caused the appearance of a punctuate fluorescence pattern in EW7MAD1 but not in EW7PC cells (figure 1B), confirming again that TNFα induces an increase in autophagic structures only in the absence of NF-κB activation. Accordingly, TNFα induces a time–dependent accumulation of the LC3-II form in EW7MAD1 cells (figure1C), whereas the level of LC3 II was not significantly modified in TNFα-treated EW7PC cells as compared to untreated cells. Interestingly, TNFα-induced the accumulation of autophagic vacuoles in cells lacking NF-κB activity correlated with their susceptibility to apoptosis (Figure 1B, right). Transmission electron microscopy experiments was also used to visualize autophagic vacuoles in cells (figure 1D, a,b,c,d). Quantitation of electron micrographs reveals an increase in the number of autophagic vacuoles after 4h TNFα treatment of cells lacking NF-κB activity as compared to EW7PC cells. The presence of autophagosomes containing mitochondrion (figure 1D, e) and degraded cytoplasmic materials was confirmed by electron microscopy (figure 1D, f).

To verify that the increase in the number of autophagic vacuoles in TNFα-treated EW7MAD1 cells represents an activation of autophagic activity rather than inhibition of autophagic vacuoles/lysosome fusion, we further measured the degradation of long–lived proteins in the presence and the absence of the autophagy inhibitor, 3-MA. As shown in figure 2, the rate of 3-MA-sensitive proteolysis of long–lived proteins was increased in a time-dependent manner after TNFα treatment in EW7MAD1 cells but not in EW7PC cells. Thus, the accumulation of autophagic vacuoles observed in TNFα-treated EW7MAD1 cells results from the activation of autophagy but not from the impairment of lysosomal delivery of LC3-positive vacuoles. It is worth noting that 3-MA-sensitive proteolysis is increased in both cell lines incubated in nutrient–free medium, confirming again that both cell lines are competent for induction of autophagy.

Overall, these findings demonstrate that inhibition of NF-κB activity results in induction of autophagy in TNFα-treated EW7MAD1 cells. Such autophagic hallmarks were also observed in another clone of Ewing sarcoma cells expressing a repressor of NF-κB activation, EW7MAD2 cells after TNFα treatment (data not shown).

The **NF-κB-dependent repression of autophagy occurs also in NB4 and MCF7 cells.**
To investigate whether the NF-κB dependent regulation of autophagy observed in Ewing sarcoma may be extend to other cell lines, we examined the autophagic capacity of two additional cell lines, the promyelocytic leukemia cells (NB4) and the human breast cells (MCF7) in the presence and absence NF-κB activation. Autophagy was evaluated by either MDC staining or detection of the accumulation of the LC3-II form by Western blotting.

We firstly compared the autophagic activity in TNFα-treated NB4 cells (NB4/GFP) and in TNFα-treated NB4 cells expressing a repressor of NF-κB activation (NB4/GFP-MAD). As shown by MDC staining (figure 3A), TNFα induced a great accumulation of autophagic vacuoles only in cells lacking NF-κB activity (NB4/GFP-MAD) but not in NB4/GFP. Accordingly, TNFα treatment resulted in an increased level of the LC3-II form only in the absence of NF-κB activation (Figure, 3C). As in Ewing sarcoma cells, this accumulation of autophagic vacuoles observed in TNFα-treated NB4/GFP-MAD cells correlated with their susceptibility to apoptosis induced by TNFα (Figure 3B).

We also examined the autophagy capacity of MCF7 cells following siRNA knockdown of p65 protein, a member of NF-κB complex. The complete reduction of p65 expression by using specific siRNA (39) (figure 3E, inset) sensitized MCF7 cells to TNFα-induced apoptosis (figure 3E) and triggered accumulation of autophagic vacuoles in these cells as revealed by MDC staining (figure 3D and Figure 3E). Accordingly, TNFα induces an accumulation of LC3II form only in MCF7 treated with siRNA against p65 (figure 3F).

Overall, these data indicate that inhibition of NF-κB in both NB4 and MCF7 cells induces autophagy in response to TNFα.

**ROS are involved in TNFα-induced autophagy in cells lacking NF-κB activity**

We have previously shown that inhibition of NF-κB activity results in an increase in ROS production in TNFα-treated Ewing sarcoma cells (41). We therefore questioned whether TNFα-induced ROS production is involved in stimulation of autophagy. To this aim, we treated EW7MAD1 cells with butylated hydroxyanisole (BHA), a ROS scavenger, prior to addition of TNFα. Autophagy was evaluated by MDC or GFP-LC3 staining. As shown in figure 4A, treatment with this antioxidant reduced the accumulation of autophagic vacuoles induced by TNFα in EW7MAD1 cells. Similar results were found by using another ROS scavenger, N-butyl-α-phenyl-nitrone (BNP) (figure 4A). This indicates that the production of ROS is required for the induction of autophagy in TNFα-treated EW7MAD1 cells. To confirm more precisely the role of ROS in induction of autophagy, we further examined the effect of H2O2 addition on the modulation of autophagic activity in NF-κB-competent cells. As revealed by MDC staining, treatment with H2O2 induced an accumulation of acidic vacuoles in
EW7PC cells. The appearance of autophagic vacuoles was inhibited in the presence of both BHA and BNP (figure 4A, 4B). Similar results were found in GFP-LC3 transfected cells showing that both TNFα and H$_2$O$_2$ promote an increase in the number of autophagic structures in EW7MAD1 and EW7PC cells, respectively (figure 4B). Both responses were reduced in the presence of BHA and BNP confirming again that the stimulation of autophagy induced either by TNFα or H$_2$O$_2$ involves a ROS-dependent mechanism (figure 4A, 4B).

**Analysis of autophagy-regulated proteins expression in TNFα-treated EW7PC cells and TNFα-treated EW7MAD1 cells**

Autophagy is mediated by Atg proteins (17) and can be negatively regulated by activation of mTOR (42,43). To gain further insight into the mechanisms of induction of autophagy in cells lacking NF-κB activity, we compared the pattern of activation or expression of such autophagy-regulated proteins in TNFα-treated EW7PC cells and in TNFα-treated EW7MAD1 cells.

We first examined the activity of mTOR by analyzing the phosphorylation of two of its substrates, the p70 protein S6 kinase and the eukaryotic initiation factor 4-E-binding protein1 (4E-BP1). As shown in figure 5A, TNFα treatment of EW7PC cells resulted in an increase in the level of phosphorylation of both 4E-BP1 and p70S6 kinase proteins. In contrast, TNFα treatment of Ewing sarcoma cells lacking NF-κB activity reduced the levels of phospho-4E-BP1 and did not significantly change the level of phospho-p70 S6 kinase. These results indicate that TNFα-induced repression of mTOR activation only when NF-κB activity is inhibited, which is in accordance with the results showing that TNFα induces autophagy in EW7MAD1 cells but not in EW7PC control cells.

We further examined the expression patterns of Atg7, Atg5 and Beclin 1, three Atg proteins involved in autophagosome formation (17), and of Bcl-2, an anti-apoptotic protein which negatively regulates autophagy through its interaction with Beclin 1 (44). As shown in figure 5A, TNFα caused a strong decrease in the level of Bcl-2 protein in EW7MAD1 cells but not in EW7PC cells. Furthermore, whereas the expression level of Beclin 1 remained unchanged following TNFα treatment of EW7PC, it was upregulated in EW7MAD1 cells 4 h after TNFα addition. The levels of expression of Atg7 and Atg5 were not modified upon TNFα treatment of both cell lines. To further specify the time-course of the modulation of Beclin 1 expression by TNFα, we also examined the expression level of Beclin 1 upon short times of incubation with TNFα in both cell lines. We found that TNFα induces a rapid increase in Beclin 1 expression level in EW7MAD1 cells 0.5 h after treatment (figure 5B). Interestingly, the level of Beclin 1 expression was unchanged during the time-course of TNFα treatment of EW7PC cells. To investigate whether the TNFα-induced accumulation of Beclin 1 expression is dependent on the increased transcription of beclin 1 gene or stabilization of the protein, we
examined the expression of mRNA level of Beclin1 by performing real-time quantitative RT-PCR. As shown in figure 5B (right panel) the level of Beclin1 mRNA was not significantly modified during two hours treatment with TNFα of EW7MAD1 cells suggesting that the increase of Beclin1 protein expression in TNFα-treated EW7MAD1 cells results from a stabilization of the protein rather than from an increase in mRNA levels. In the same way, we found that the addition of H2O2 rapidly increased the expression of Beclin1 protein in EW7PC NF-κB-competent cells (figure 6B). To investigate whether these accumulations of Beclin1 protein are dependent on ROS production, we examined the effect of antioxidant. As shown in Figure 6C (left panel), pretreatment of EW7MAD1 cells with BHA markedly prevented TNFα–induced Beclin 1 expression protein. Similarly, H2O2-induced upregulation of Beclin1 protein was reduced by the addition of BHA (Figure 5C, right panel). Collectively, these data show that both TNFα and H2O2 cause a rapid ROS-dependent increase in Beclin 1 expression prior to the accumulation of autophagic vacuoles.

**Activation of autophagy participates in the apoptotic signaling triggered by TNFα in EW7MAD1 cells**

To investigate the role of autophagy in TNFα-induced apoptosis in EW7MAD1 cells, we knocked down the expression of beclin1 and Atg7 by using specific siRNAs. As shown in figure 6A, these treatments resulted in an inhibition of TNFα-induced autophagy in EW7MAD1 cells. Apoptosis was evaluated by Hoechst staining, detection of the cleaved form of PARP1 and quantification of nucleosomal DNA fragmentation in cells. As shown in figure 6B, the inhibition of both beclin1 and atg7 expression by specific siRNA reduced the accumulation of apoptotic nuclei (condensed and fragmented nuclei) in TNFα-treated EW7MAD1 cells (Figure 6B, left and right). Furthermore, TNFα-mediated PARP1 cleavage in TNFα-treated EW7MAD1 was reduced after knock down of beclin1 and atg7 (Figure 6C, left). Apoptosis was also quantified by using DNA fragmentation Elisa assay. As shown in figure 6C (right), TNFα–induced nucleosomal DNA fragmentation in EW7MAD1 was markedly decreased following inhibition of autophagy by using beclin1 and atg7 siRNAs.

Altogether, these findings show that inhibition of autophagy reduces TNFα-induced apoptosis in EW7MAD1 cells, suggesting that autophagy is involved in the apoptotic pathway under these conditions. Similarly, H2O2-induced apoptosis in EW7PC cells was reduced following inhibition of autophagy by using siRNA directed against beclin1 and atg7, indicating that autophagy is also implicate in the signaling pathway leading to ROS-induced apoptosis (figure 6D). Interestingly, inhibition of autophagy by using specific siRNA also reduced TNFα–induced ROS production in EW7MAD1 cells (data not shown). These observations are in accordance with previous report supporting that autophagy
contributes in the regulation of cellular ROS production (45).

**Caspase activation inhibits TNFα-induced autophagy in EW7MAD1 cells**

Since the treatment of EW7MAD1 cells with TNFα elicits caspase-dependent cell death (34,41), we further examined whether caspases modulate TNFα-induced autophagy. We treated cells with Z-VAD-fmk, a broad-spectrum caspase inhibitor, prior to the addition of TNFα. Autophagy was evaluated by either MDC staining or detection of the accumulation of the LC3-II form by Western blotting. As previously shown (34), Z-VAD-fmk completely inhibited TNFα–induced apoptosis in these cells (data not shown). As shown by MDC staining, the addition of Z-VAD-fmk prior to TNFα treatment increased the number and size of autophagic vacuoles as compared to TNFα alone (figure 7A). Similarly, the accumulation of the LC3-II form induced by TNFα was enhanced following addition of Z-VAD-fmk (figure 7B). These results indicate that caspase activation downregulates autophagy.

**DISCUSSION**

NF-κB is most commonly, considered as a mediator of tumor promotion based on its ability to promote cell survival, enhance cell proliferation and decrease the sensitivity of cancer cells to apoptosis (1,2,10,46). Studies focused on identifying the mechanisms involved in the anti-apoptotic functions of NF-κB (46) have demonstrated that its activation results in an increased expression of several anti-apoptotic proteins such as Bcl-2 family members (6,47) or caspase inhibitory proteins (7). Another anti-apoptotic mechanism associated with the activation of NF-κB involves the impairment of both prolonged activation of JNK (41,48,49) and ROS production through upregulation of antioxidant proteins (41,50-53).

The present study uncovers a novel anti-apoptotic function of NF-κB activation that consists in repression of autophagy. We show that whereas TNFα induces the stimulation of autophagy in Ewing sarcoma cells lacking NF-κB activity, it does not activate this process in Ewing sarcoma NF-κB-competent cells. Similar results were observed in another clone of Ewing sarcoma cells and NB4 cells carrying a repressor for NF-κB activation as well as in MCF7 cells following inhibition of p65 expression by using specific siRNA. These findings support the conclusion that the NF-κB-dependent inhibition of autophagy may be a general cellular response.

Although the implication of autophagy in nonapoptotic programmed cell death, known as autophagic cell death (54-56), has been pointed out in several studies, its contribution to apoptosis is less clear. Here, we show that siRNA-mediated knock down of autophagy-related genes reduces TNFα-induced apoptosis in cells lacking NF-κB activation. These findings contradicted by results showing that autophagy is an anti-apoptotic mechanism under certain stress conditions (25,30,57). Thus, depending on cellular context,
autophagy may have pro-apoptotic or anti-apoptotic functions. The molecular mechanisms that determine the switch between these two responses remain to be elucidated.

The role of caspase in the modulation of autophagy activity has been pointed out in few recent studies (26,32). Here, we found that the inhibition of caspase activation by Z-VAD-fmk—which totally inhibits TNFα–induced apoptosis—enhances TNFα-mediated stimulation of autophagy in cells lacking NF-κB activation. This suggests that the activation of caspases can in turn control the initial autophagic activity. The negative regulation of autophagic activity by caspases has also been reported by Xue et al. who showed that blockade of caspase activities induces an autophagy-related sequestration of mitochondria in Nerve Growth Factor-deprived neuroblastoma cells (58). Recently, another report (32) demonstrates that autophagy-related cell death is induced by caspase 8 inactivation. Hence, our findings, together with these results support the notion that autophagy and apoptosis may regulate each other.

We and others have previously shown that impairment of NF-κB activation results in accumulation of ROS in several cell lines in response to distinct activators of NF-κB (41,48,49). The role of ROS in autophagy stimulation has been shown by using oxidative stress conditions such as treatment of neuroblastoma cells with dopamine (57), hyperoxia (59) and in Syrian hamster Harderian gland, a physiological model of oxidative stress (60). Nevertheless, direct evidence that ROS induces autophagy has been lacking. In the present report, we demonstrate that ROS participate in TNFα–induced stimulation of autophagy in cells carrying a repressor of NF-κB and that direct addition of exogenous H₂O₂ to NF-κB-competent cells is also able to induce autophagy. Furthermore, we found that TNFα and H₂O₂ rapidly induce Beclin 1 expression in EW7MAD1 cells and EW7PC cells, respectively. In both cases, the increase in Beclin 1 expression precedes the accumulation of autophagic vacuoles. Moreover, the antioxidants BHA and BNP markedly prevent the increase in Beclin 1 expression and autophagy, suggesting that ROS are involved in these processes. It has been reported that the level of expression of Bcl-2 plays a role in the regulation of autophagy through its interaction with Beclin 1 (44,61). Hence, our observation that TNFα mediates downregulation of Bcl-2 protein in cells lacking NF-κB activation argues for a possible mechanism by which autophagy may be stimulated in these cells.

The mTOR pathway has been shown to regulate autophagy negatively (19,43). Here, we provide evidence that TNFα upregulates mTOR activity in an NF-κB-dependent manner, since this activity is induced in TNFα-treated NF-κB competent cells and, inversely, impaired in TNFα–treated cells lacking NF-κB activity. These results are consistent with the opposite autophagic capacities observed in these two cell lines after treatment with TNFα. Nevertheless, the
molecular mechanisms by which NF-κB activity regulates mTOR activity need more extensive investigations.

In conclusion, we demonstrate that NF-κB-mediated repression of autophagy may constitute a novel anti-apoptotic mechanism of this transcription factor. This suggests that activation of autophagy is a potential way of bypassing the resistance of cancer cells to anticancer agents that activate NF-κB.

**FOOTNOTES**

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** During the review of this manuscript, the role of ROS in the induction of autophagy in macrophages has been reported (Xu & al. 2006 JBC published online May 15)

1Abbreviations used: NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; IκB, inhibitor κB, PARP; poly (ADP-ribose) polymerase; ROS, reactive oxygen species; TNFα, tumor necrosis factorα; FADD, Fas-associated death domain protein; Trail, tumor necrosis factor (TNF)-related apoptosis-inducing ligand; 4E-BP1, eIF4E-binding protein-1; Atg, Autophagy-related gene; (m)TOR, (mammalian) Target Of Rapamycin; BHA, butylated hydroxyanisole; BNP, N-butyl-α-phenyl-nitrone; MDC, monodansylcadaverine; small interfering RNA (siRNA); 3-MA, 3-methyladenine.

**FIGURE LEGENDS**

Fig. 1. TNFα stimulates autophagy in Ewing sarcoma cells expressing a repressor of NF-κB activation

A) EW7PC and EW7MAD1 cells were treated with TNFα (2000 Unit/ml) for different times or were incubated for 4 h in nutrient–free medium (NF). Cells were stained with MDC as detailed in Materials and Methods. Left. Representative images of three independent experiments. The bar represents 6 μm. Right, the percentage of cells with MDC staining dots per total MDC staining cells was quantified after treatment with either TNFα (8h) or nutrient–free medium treatment 4h.. Values reported are the mean ± SD of three independent experiments.

B) Left. Representative images of GFP-LC3 staining in EW7PC and EW7MAD1 cells incubated for 8 h in the presence and absence of TNFα. The bar represents 3 μm. Right, the percentage of cells with
punctuated GFP-LC3 per total GFP-LC3 cells was scored. Values are the mean ± SD of three independent experiments. For each condition, cells are also subjected to Hoechst staining and the percentage of apoptotic nuclei was scored.

C) Left. Immunoblot analysis of LC3-I processing into LC3-II in EW7PC and EW7MAD1 cells treated with TNFα for the indicated times. Right, bands corresponding to LC3-II (at time-point 8 h) were quantified using NIH Image software and were normalized to actin expression level.

D) Left. (a,b,c,d) Representative electron micrographs of EW7PC and EW7MAD1 cells treated for 4 h in the presence and absence of TNFα are shown. Arrowheads denote the presence of autophagic vacuoles. The bar represents 0.5 μm. (e,f) Higher magnifications of TNFα-treated EW7MAD1 cells show autophagosomes containing mitochondrion (e) or digested cytoplasmic materials (f). The bar represents 0.2 μM. Right, the total number of autophagic vacuoles per cell profile was determined for each condition. Results shown in D are the mean ± SD of 5 profiles for each condition.

Fig. 2. The effect of TNFα on long-lived protein degradation in EW7PC and EW7MAD1 cells
The rate of proteolysis of [14C] valine-labeled long-lived proteins was measured in cells following 8 h incubation with TNFα in the presence and absence of 10 mM 3-methyladenine (3-MA). As a positive control, EW7MAD1 cells were subjected to 4 h starvation in the presence and absence of 10 mM 3-MA. Results are the mean ± SD of three independent experiments.

Fig. 3. Inactivation of NF-κB in NB4 and MCF7 cells induces autophagy in response to TNFα.
NF-κB-competent NB4 cells (NB4/GFP), NB4 cells expressing an inhibitor of NF-κB activation (NB4/GFP-MAD), MCF7 cells and MCF7 cells transiently transfected with siRNA against p65 were treated with TNFα (2000 Unit/ml) for different times as indicated.
A,D) Representative control cells and TNFα-treated cells labelled with MDC are shown. The bar represents 3μm.
B,E) The percentage of cells with MDC stained dots was quantified after treatment with TNFα of NB4 cells or MCF7 cells, for 8h or 32h, respectively (black bar). Inset, the levels of p65 protein expression in presence and absence of p65 siRNA were shown. For each condition, cells are also subjected to Hoechst staining and the percentage of apoptotic nuclei was scored (white bar).
C,F. Immunoblot analysis of LC3-I processing into LC3-II in NB4/GFP and NB4/GFP-MAD cells treated in the presence and absence of TNFα (C) and in MCF7 cells incubated for 32h with siRNA against p65 before addition of TNFα (F).

Fig. 4. ROS scavengers inhibit TNFα-induced autophagy in EW7MAD1 cells
EW7MAD1 and EW7PC cells were treated with either BHA (100 μM) or BNP (50 μM) for 2 h prior treatment with TNFα (2000 U/ml, 8 h) and H2O2 (100 μM, 8 h), respectively.
A) Fluorescence microscopy was performed following MDC staining. Representative images of cells with MDC staining are shown. The bar represents 6 µm.

B) EW7PC and EW7MAD cells were transfected with GFP-LC3 plasmids and then subjected to treatment as indicated in A. For each condition, the percentage of cells with punctuate GFP-LC3 staining was quantified. Results shown in A and B are representative of three independent experiments.

**Fig. 5. Comparison of autophagy-regulated protein expression in TNFα-treated EW7PC cells and in TNFα-treated EW7MAD cells**

(A) EW7PC and EW7MAD1 cells were treated for the indicated times with TNFα before cell lysis. Equal protein amounts of whole cell extracts were analyzed by Western blotting using antibodies directed against phospho-4EBP1, 4EBP1, phospho-p70 S6kinase, p70 S6kinase, Beclin 1, Atg7 and Atg5, Bcl-2, actin.

B) EW7PC and EW7MAD1 cells were treated for the indicated times with either TNFα (2000 units/ml) or H2O2 (100 µM).

*Left.* Cellular extracts were prepared and analyzed for the expression level of Beclin 1. The bands corresponding to Beclin 1 protein level in EW7MAD1 cells were quantified using NIH Image software and were normalized to actin expression level (*right*). *Right.* Total RNA was extracted from TNFα-treated EW7MAD1 cells before the measurement of the Beclin 1 mRNA level by real-time quantitative RT-PCR. Values obtained for Beclin 1 mRNA transcripts were normalised to those of β actin.

C) EW7MAD1 and EW7PC cells were first treated in with BHA (100 µM) and then incubated with TNFα (2000 units/ml, 1h) or H2O2 (100 µM, 0.5h), respectively. Cellular extracts were prepared and analyzed for Beclin 1 expression by Western Blotting.

Results shown in A, B, C are representative of three independent experiments.

**Fig. 6 Effect of autophagy inhibition on TNFα-induced apoptosis in EW7MAD1 cells**

EW7MAD1 cells were transiently transfected with beclin 1 siRNA, atg7 siRNA or control siRNA. TNFα (2000 U/ml, 8 h) was added 54 h later.

A) *Left.* Cell extracts were analyzed by Western blotting using antibodies against Beclin 1, Atg7. *Right*, the percentage of cells with punctuated GFP-LC3 per total GFP-LC3 cells was scored. Values are the mean ± SD of two independent experiments.

B) *Left.* The percentage of cells carrying an apoptotic nuclei was determined by Hoechst staining. Values are the mean ± SD of four independent experiments. *Right*, representative cells are shown and arrowheads indicate apoptotic nuclei (condensed and fragmented nuclei).
C)  *Left.* Cellular extracts were analyzed by Western blotting using antibodies against PARP1 and actin. *Right,* apoptosis was measured by quantification of nucleosomal DNA fragmentation. The ratio of DNA fragmentation in TNFα–treated cells to that in untreated cells were determined. Results are representative of three independent experiments.

D)  EW7PC cells were transiently transfected with beclin 1 siRNA, atg7 siRNA or control siRNA. H2O2 (100 µM, 8 h) was added 54 h after transfection. The percentage of cells carrying an apoptotic nucleus was determined by Hoescht staining.

**Fig. 7. Effect of caspases inhibition on TNFα - induced autophagy in EW7MAD1 cells**

EW7MAD1 cells were first incubated for 2 h with Z-VAD-fmk (50 µM) and then treated for 8 h with TNFα. The bar represents 6 µm.

A)  Cells were subjected to MDC staining before analysis by fluorescence microscopy.

B)  Cellular extracts were prepared for Western blotting analysis of LC3-II expression. Results shown in A and B are representative of three independent experiments.
REFERENCES


Fig. 1

A) Time after TNFα treatment (h)

EW7 PC

EW7 MAD1

B) Control TNFα

EW7 PC

EW7 MAD1

C) EW7PC EW7MAD1

Time (h) 0 4 8 16 LC3-I LC3-II Actin

D) Control TNFα

EW7 PC

EW7 MAD1

Number of autophagic vacuoles per cell profile

control TNFα
Fig. 2
**Fig. 3**

**A)**
- Control
- TNFα (8h)

**B)**
- NB4/GFP
- NB4/GFP-MAD

**C)**
- Time (h)
- TNFα: 0, 4, 8

**D)**
- MCF7
- Control
- TNFα (32h)
- siRNA p65
- TNFα (32h) + siRNA p65

**E)**
- Punctuated MDC staining cells (%)
- Control siRNA
- p65 siRNA
- TNFα siRNA

**F)**
- Time (h): 0, 16, 32
- LC3-I
- LC3-II
- Actin
Fig. 4

A) Control  TNFα  TNFα + BHA  TNFα + BNP
EW7MAD1

Control  H₂O₂  H₂O₂ + BHA  H₂O₂ + BNP
EW7PC

B) Punctuated GFP-LC3 cells per total GFP-LC3 cells (%)

EW7MAD1  EW7PC

Control  TNFα  TNFα + BHA  TNFα + BNP
Control  H₂O₂  H₂O₂ + BHA  H₂O₂ + BNP
**A)**

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**Phospho-4EBP1**

**4EBP1**

**Phospho-p70**

**p70**

**Bcl-2**

**Beclin 1**

**Atg7**

**Atg5**

**Actin**

---

**B)**

**Beclin 1**

**Actin**

**EW7PC**

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**Beclin1**

**Actin**

---

**C)**

**Beclin1**

**Actin**

**EW7MAD1**

**EW7PC**

---

*Fig. 5*
Fig. 6

A) Beclin 1

Actin

Atg7

Actin

B) % of apoptotic nucleus

C) DNA fragmentation (fold induction)

D) % of apoptotic nucleus
A)

Control

TNFα

Z-VAD-fmk

TNFα + Z-VAD-fmk

B)

LC3-I →

LC3-II →

actin →

control

TNFα

TNFα + Z-VAD-fmk

Z-VAD-fmk

Fig. 7
NF-kappa B activation represses TNF alpha-induced autophagy
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Chantal Bauvy, Sylvie Souquère, Gérard Pierron and Patrice Codogno

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