Resistance of bone marrow-derived macrophages to apoptosis is associated with the expression of X-linked inhibitor of apoptosis protein in primary cultures of bone marrow cells

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In this study we investigated the underlying mechanisms that confer resistance on mature macrophages with the use of macrophage colony-stimulating factor (M-CSF)-induced bone marrow-derived macrophages (BMDM). In the presence of M-CSF, immature precursor cells were induced to undergo proliferation and differentiation into mature macrophages *in vitro* with cell morphology similar to that of tissue macrophages by day 7–10. Immunoblot analyses showed that bone marrow precursors express appreciable levels of caspase-3 and caspase-9 but no or very low levels of c-*fms* (M-CSF receptor) and the apoptosis regulators X-linked inhibitor of apoptosis protein (XIAP), c-IAP-1, Bcl-2 and Bax. The differentiation of BMDM is associated with a steady and gradual increase in the levels of c-*fms*, XIAP, c-IAP-1, Bcl-2 and Bax, reaching maximal levels by day 7. However, the levels of caspase-3 and caspase-9 stayed essentially unchanged even after prolonged incubation (more than 10 days) with M-CSF. Unlike bone marrow precursor cells, mature BMDM (day 7–10) were resistant to apoptosis induced by M-CSF depletion, which includes the activation of caspase-3 and caspase-9 and the degradation of XIAP, Bcl-2 and Bax proteins in the process. Treatment of day 7 BMDM with XIAP anti-sense oligonucleotides (oligos), but not sense oligos, partly abolished their resistance to apoptosis. By using a gel-shift assay and a specific nuclear factor κB (NF-κB) inhibitor, we demonstrated that NF-κB activity is responsible for the up-regulation of XIAP in M-CSF-treated macrophages. In addition, treatment of starved macrophages with M-CSF induced a rapid phosphorylation of Akt kinase before the activation of NF-κB. Our results showed that XIAP is one of the anti-apoptotic regulators that confer resistance on mature macrophages by M-CSF.

Key words: caspase, differentiation, macrophage, M-CSF, nuclear factor-κB.

INTRODUCTION

Macrophages are ubiquitous cells with central roles in the innate immune responses. They are important accessory cells for many immune reactions that are crucial in the defence against invading pathogens. Tissue macrophages are derived from bone marrow myeloid stem cells via blood monocytes by complicated processes involving both cell replication and differentiation. A group of haemopoietic growth factors are required for the growth and production of mature end-cells from primary cultures of bone marrow cells. Among this group of growth factors, macrophage colony-stimulating factor (M-CSF, also known as CSF-1) is the growth factor that acts primarily on bone marrow precursors committed to the monocytic lineage [1,2]. In addition, M-CSF can prolong the survival of monocytes and enhances their functional activities *in vitro* [3]. The biological effects of M-CSF are mediated through a unique tyrosine kinase receptor, M-CSF receptor (M-CSFR), encoded by the proto-oncogene c-*fms*, which is the cellular counterpart of the v-*fms* oncogene [4]. The presence of macrophage progenitors is demonstrated in semi-solid agar cultures of bone marrow cells containing M-CSF in which immature precursor cells are induced to undergo extensive proliferation and differentiation, giving rise to macroscopic colonies composed of differentiated macrophages after 7–10 days in culture [5].

Apoptosis, or programmed cell death, is a normal physiological cell suicide process that is evolutionarily conserved in all animals [6]. The execution of cellular apoptosis involves the activation of a cascade of intracellular cysteine proteases known as caspases. Caspases are initially synthesized as inactive proenzymes; activation involves the proteolytic cleavage of proenzymes into smaller active subunits. A number of downstream effector caspases cleave specific cellular substrate proteins and mediate the deliberate disassembly of the cell into apoptotic bodies, facilitating the demise of the cell [7].

Other controllers of apoptosis are the Bcl-2 family proteins, which function upstream of caspases by either promoting or suppressing their protease activities. Bcl-2 and Bax are the two prototype members of this large family of proteins, with Bcl-2 being the apoptosis inhibitor and Bax the apoptosis promoter. Although the detailed mechanism is unknown, it is thought that they form protein dimers with each other; the relative ratio of these two proteins can determine the balance between life and death [8,9]. Recent studies have revealed yet another group of intracellular proteins that negatively regulate apoptosis execution, primarily by interfering with the caspase cascade. Proteins of the inhibitor of apoptosis (IAP) family are a group of anti-apoptotic proteins that are highly conserved across several species [10]. They can block apoptotic events by directly binding and inhibiting selected caspases. So far at least six human IAP

Abbreviations used: BMDM, bone marrow-derived macrophages; CAPE, caffeic acid phenylethyl ester; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; IAP, inhibitor of apoptosis; IκB; inhibitory κB; M-CSF, macrophage colony-stimulating factor; M-CSFR, M-CSF receptor; NF-κB, nuclear factor κB; oligos, oligonucleotides; PEM, peritoneal exudate macrophage; XIAP, X-linked inhibitor of apoptosis protein.

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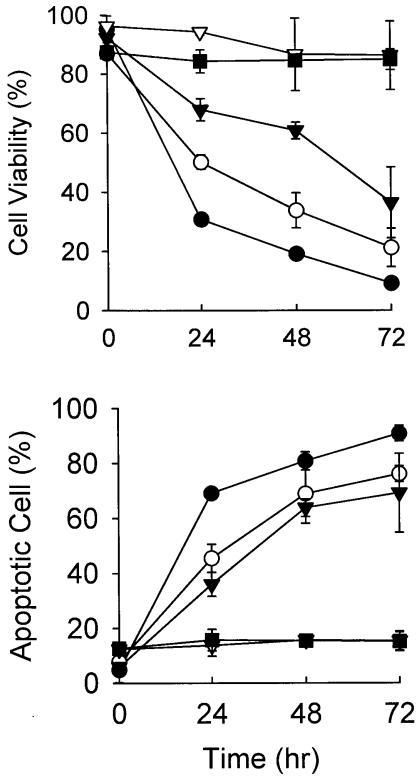

Figure 1 Induction of apoptosis in BMDM and PEM cells by M-CSF depletion

Bone marrow non-adherent cells were cultured in the presence of M-CSF (10 ng/ml) for various periods. At the times indicated, cells were removed, washed and cultured in M-CSF-free medium for 24, 48 and 72 h. Upper panel: at the end of the incubation period, cell viability was determined with the Trypan Blue uptake method. Lower panel: apoptotic cell numberss were determined by morphological examination after staining with Acridine Orange. For each sample, 200 cells were counted. Data are means ± S.D. for duplicate experiments. Note the resistance to apoptotic cell death by day 7 adherent cells and PEM cells after the removal of M-CSF. Symbols: ●, day 0 non-adherent cells; ○, day 2 non-adherent cells; ▼, day 2 adherent cells; ▽, day 7 adherent cells; ■, PEM cells.

family proteins have been described [NAIP, cIAP-1, cIAP-2, X-linked inhibitor of apoptosis protein (XIAP), survivin and BIR repeat-containing ubiquitin-conjugating enzyme ('BRUCE')] [11–15].

A potent mammalian IAP is XIAP, for which the mechanism of action involves the direct inhibition of caspase-3 and caspase-7, two key effector proteases of the apoptosis pathway [13,16]. Wagenknecht et al. [17] showed that overexpression of XIAP, but of not cIAP-1 or cIAP-2, can block caspase-3 processing and prevent apoptosis in LN-18 and LN-229 glioma cells. Xu et al. [18], in contrast, reported that the overexpression of XIAP in CA1 neurons can prevent apoptosis and allow proper functions after ischaemic insult in these cells. Results from other studies suggest that the expression of IAP genes might be under the regulation of nuclear transcription factor κB (NF-κB). Transcription factors that comprise the NF-κB/Rel family are important regulators of immune and inflammatory responses [19,20]. A critical step in the control of NF-κB is the site-specific phosphorylation of its inhibitor, inhibitory κB (IκB), which targets the latter for degradation by the ubiquitin–proteasome pathway [21]. NF-κB is involved in conferring resistance to apoptosis induced by various stimuli [22–24]. Up-regulation of cIAP-1 and cIAP-2 might offer one explanation for the anti-apoptosis effect associated with NF-κB activation [25,26].

The survival of bone marrow stem cells *in vitro* is strictly dependent on exogenous growth factors. However, tissue macrophages can sustain a much longer life span *in vitro* without the addition of exogenous growth factors [27]. In the present study we examined the importance of XIAP and the development of anti-apoptotic mechanisms during macrophage differentiation in a primary cell model of bone marrow-derived macrophages (BMDM). We now report that macrophage differentiation is associated with an enhanced expression of an anti-apoptotic protein, XIAP, which confers on mature macrophages the ability to resist apoptosis induced by M-CSF depletion.

MATERIALS AND METHODS

Reagents

Recombinant human M-CSF was generously provided by Cetus Corp. (Emeryvill, CA, U.S.A.). FBS (fetal bovine serum) was purchased from HyClone Laboratory. Anti-(human XIAP) antibody was a product of Medical and Biological Laboratories Co. (Medical and Biological Laboratories, Naka-ku, Nagoya, Japan). Anti-(Bcl-2), anti-(caspase-3) and anti-Bax antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-(caspase-9) antibody was purchased from PharMingen (San Diego, CA, U.S.A.). Akt and phospho-Akt (Ser473) antibodies were products of New England BioLabs (Beverly, MA, U.S.A.). Caspase-9/Mch6 and caspase-3 colorimetric protease assay kits were purchased from MBL and CalBioChem Co. (San Diego, CA, U.S.A.) respectively. Second-generation anti-sense oligonucleotides (oligos) and phosphorothioate sense oligonucleotides were synthesized by Oligos Etc. (Wilsonville, OR, U.S.A.). Oligomer sequences were based on the published mouse X-linked inhibitor of apoptosis (miap-3) mRNA sequence [28], which is near the N-terminal translation initiation site of miap-3. The sequences were as follows: 5′-CCT TCA AAA CTG TTA AA-3′ (anti-sense), 5′-TTT AAC AGT TTT GAA GG-3′ (sense) and 5′-ATC TTC AAC TAG TAC AA-3′ (scramble). Oligomers were dissolved in water at a concentration of 10 mM and kept at −70 °C until needed. Caffeic acid phenylethyl ester (CAPE) was obtained from Alexis Biochemicals (San Diego, CA, U.S.A.).

Collection of mouse bone marrow cells

Female DBA/2J mice 6–8 weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME, U.S.A.). Mouse bone marrow cells were collected from femoral shafts by flushing with 3 ml of cold RPMI-1640 containing 15% (v/v) FBS [29–31]. The cell suspensions were passed up and down six times through an 18-gauge needle in RPMI-1640 to disperse cell clumps. Adherent bone marrow cells were removed by incubation at 37 °C for 4 h in T-75 Falcon culture flasks. The non-adherent bone marrow cells were then cultured in RPMI-1640 containing 15% (v/v) FBS and 10 ng/ml M-CSF. Peritoneal exudate macrophage (PEM) cells were harvested by peritoneal lavage with 5 ml of RPMI-1640 3 days after one intraperitoneal injection of 1.5 ml of fluid thioglycollate medium (Difco Laboratories, Detroit, MI, U.S.A.). The yield of exudate cells varied from 10^7 to 2.5×10^7 cells per lavage, of which more than 60% were identified as mononuclear phagocytes. Non-adherent exudate cells were removed by repeated washes after incubation of the cells at 37 °C for 2 h in T-25 flasks or 35 mm culture dishes.

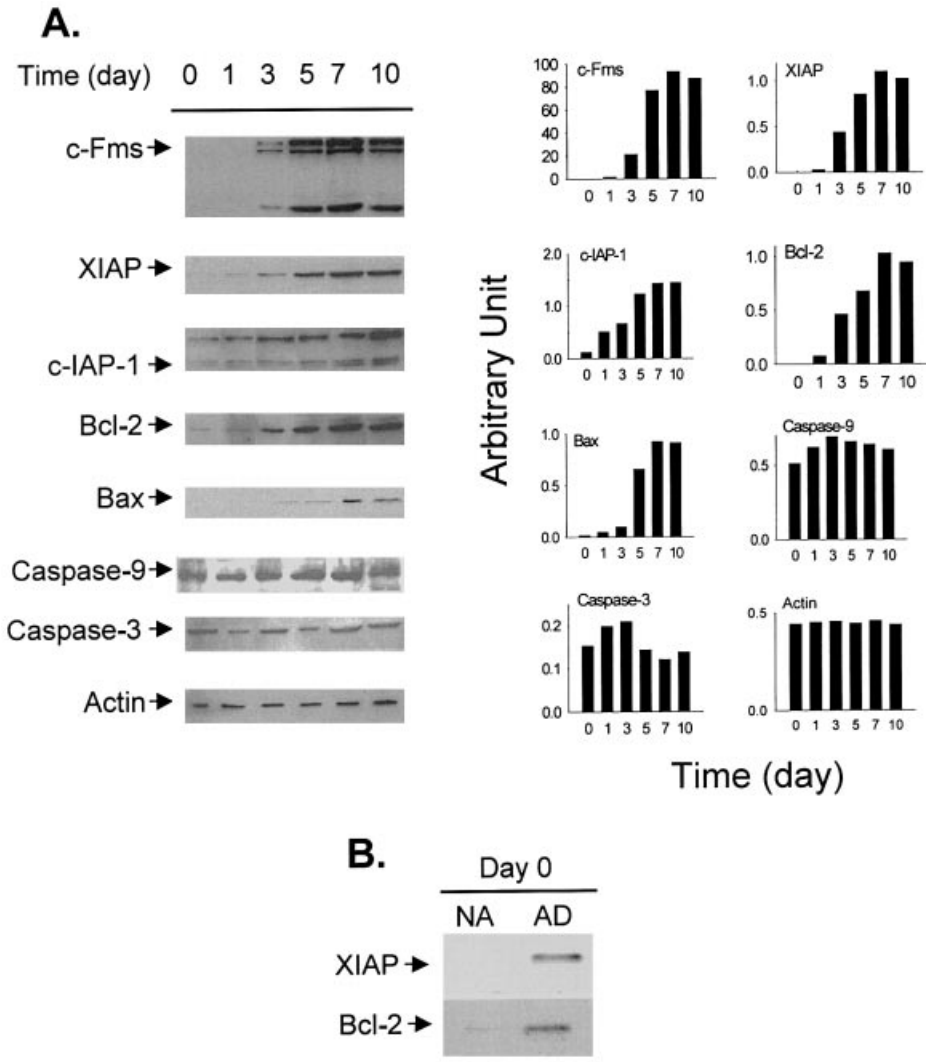

Figure 2 Expression of XIAP and apoptosis-related proteins during differentiation in BMDM

(**A**) Bone marrow non-adherent cells were cultured in the presence of M-CSF (10 ng/ml) for various periods. At the times indicated, adherent cells were removed from the cultures, counted, washed and lysed with SDS sample buffer. Samples were subjected to SDS/PAGE and immunoblot analyses with individual antibodies against c-Fms, XIAP, cIAP-1, Bcl-2, Bax, caspase-9, caspase-3 and actin (left panel). Densitometry readings of the blots are expressed in arbitrary units (right panel). (**B**) Bone marrow adherent (AD) cells and non-adherent (NA) cells from the adherence selection step of bone marrow collection were counted, washed and lysed with SDS sample buffer. Samples were subjected to SDS/PAGE and immunoblot analyses as above. The expression of XIAP and Bcl-2 was detected.

Caspase activity assay

The measurement of caspase-9/Mch6 or caspase-3 activity was performed exactly as described previously [32]. A_{405} of samples was read in a spectrophotometer with a 100 μl quartz cuvette. Control cultures without cell lysates were used as controls. Fold increases in caspase-9 or caspase-3 activities were determined by comparison with the level of controls.

Western blot analysis

After protein determination, total cell lysates (4×10^5 cells) were boiled in 10 μl of SDS gel-loading buffer for 10 min. The samples were subjected to one-dimensional SDS/PAGE. After electrophoresis, proteins were transferred to a 0.2 μm pore-size nitrocellulose membrane (Schleicher & Shuell, Keene, NH, U.S.A.) at 4 °C and 14 V overnight or at 40 V for 2 h. Non-specific binding sites on the nitrocellulose membrane were blocked by incubation in blocking buffer [5 % (w/v) non-fat dried milk] for 2 h at room temperature or overnight at 4 °C. The blots were washed once with Tris-buffered saline [10 mM Tris/HCl (pH 7.5)/150 mM NaCl] and incubated for 2 h at room temperature with the primary antibody. After the removal of excess primary antibody with three washes, the blots were incubated with a secondary antibody (goat anti-mouse or goat anti-rabbit antibodies conjugated with horseradish peroxidase). The membrane was developed with enhanced chemiluminescence reagent and exposed to Hyperfilm-ECL® (Amersham Life Science Corp.) for detection.

Electrophoretic mobility-shift assay (EMSA)

DNA-binding protein extractions were performed as described in detail previously [33]. In brief, 5×10^6 cells were washed once with cold PBS and suspended in 400 μl cold lysis buffer [10 mM

Hepes/KOH (pH 7.9)/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol/1 × protease inhibitor cocktail] with constant flicking of the tube. The cells were allowed to swell on ice for 15 min, then vortex-mixed vigorously for 10 s. The homogenate was centrifuged at 3000 ***g*** for 30 s; the supernatant fraction was discarded. The nuclear pellet was resuspended in 50 μl of ice-cold nuclear extraction buffer [20 mM Hepes/KOH (pH 7.9)/25 % (v/v) glycerol/420 mM NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM dithiothreitol/1 × protease inhibitor cocktail] and incubated on ice for 20 min for high-salt extraction. The mixture was centrifuged for 5 min at 4 °C; the supernatant (nuclear extract) was either used immediately or stored at −70 °C.

EMSA was performed by incubating 5 μg of nuclear extract with ^{32}P-labelled NF-κB p50 oligos, 5′-GCC ATG GGG GGA TCC CCG AAG TCC-3′ (Geneka Biotechnology, Montreal, Canada) for 25 min at room temperature in reaction buffer [10 mM Tris/HCl (pH 7.5)/50 mM NaCl/1mM dithiothreitol/1 mM EDTA/5 % (v/v) glycerol]. The DNA–protein complexes formed were separated from free oligos on 4 % (w/v) native polyacrylamide gel containing 50 mM Tris/HCl, pH 7.5, 0.38 M glycine and 2 mM EDTA. The gel was dried and bands were detected by autoradiography.

RESULTS

Induction of apoptosis in BMDM by withdrawal of exogenous M-CSF

After the removal of adherent cells, non-adherent bone marrow cells containing macrophage progenitor cells were cultured in growth medium containing M-CSF for various periods. In the presence of M-CSF, non-adherent bone marrow cells began to proliferate, with a fraction of them being rapidly transformed into adherent cells in as little as 12 h. After 72 h of incubation, more than 50 % of the cells in cultures were adherent cells with monocyte/macrophage morphology. They continued to replicate and grow into mature macrophages with distinct pseudopodia and a cytoplasmic vacuole. By day 7, over 95 % of the total cells in cultures were differentiated, mature macrophages. The survival of the bone marrow-derived cells obtained from various differentiation stages was examined. Cells were washed extensively with warm medium to remove exogenous M-CSF, after which they were cultured without M-CSF for various periods. As shown in Figure 1 (upper panel), freshly obtained non-adherent bone marrow cells were the most vulnerable; in the absence of exogenous M-CSF for 72 h more than 95 % of them were dead. Morphological examinations after staining with Acridine Orange showed that cell death was mediated through an apoptotic mechanism (Figure 1, lower panel). Immature macrophages obtained from day 2 cultures also were highly dependent on M-CSF for survival. In contrast, more than 80 % of the differentiated macrophages from day 7 cultures were still alive after the removal of M-CSF for the same period (Figure 1, upper panel). Similarly, PEM cells were highly resistant to apoptosis in cultures without M-CSF.

Development of anti-apoptotic mechanism during macrophage differentiation

The preceding experiments showed that progenitor cells in the bone marrow are highly dependent on exogenous growth factors for their survival and growth. To gain insight into the mechanisms used by mature macrophages to protect themselves from undergoing apoptosis, we studied the expression of a number of apoptosis-related gene products (XIAP, c-IAP-1, Bcl-2, Bax, caspase-3 and caspase-9) in BMDM obtained from different stages of differentiation. As shown in Figure 2(A), immature macrophage progenitor cells, including day 1 immature adherent cells, displayed very low levels of c-*fms* product, a macrophage-specific marker, and four apoptosis-related gene products XIAP, c-IAP-1, Bcl-2 and Bax. The levels of these products increased steadily during the culture periods. From day 1 to day 10 the levels of XIAP, c-IAP-1, Bcl-2 and Bax along with c-*fms* product increased severalfold as the cells became differentiated (Figure 2A). In contrast, high levels of caspase-3 and caspase-9 were detected in freshly obtained bone marrow cells as well as in immature day 1 adherent cells. Further, no significant changes in the levels of caspase-3 and caspase-9 were noticed during the differentiation of BMDM from day 1 to day 10. Because adherent bone marrow cells obtained in the adherence selection step contain, at least in part, monocytes in several stages of differentiation, we analysed the expression of XIAP and Bcl-2 in this cell population. As shown in Figure 2(B), high levels of XIAP and Bcl-2 were detected in adherent cells but not in non-adherent cells.

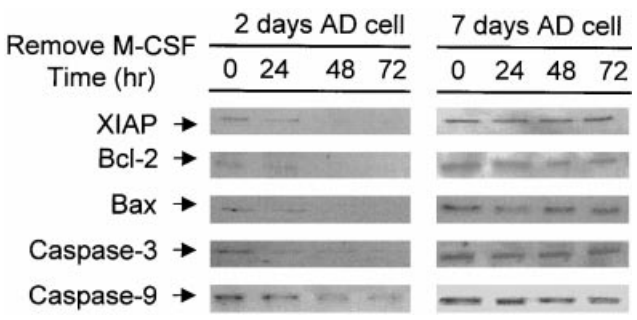

Figure 3 Effect of M-CSF removal on the expression of XIAP, Bcl-2, Bax and caspases

After extensive washes, day 2 and day 7 BMDM were cultured in the absence of M-CSF for various periods. At the times indicated, cells were removed, washed and lysed in SDS sample buffer. Cell lysates were subjected to immunoblot analyses with antibodies against XIAP, Bcl-2, Bax, caspase-9 and caspase-3. Note the degradation of XIAP, Bcl-2, Bax and caspases in day 2 BMDM after depletion of M-CSF. Abbreviation: AD, adherent.

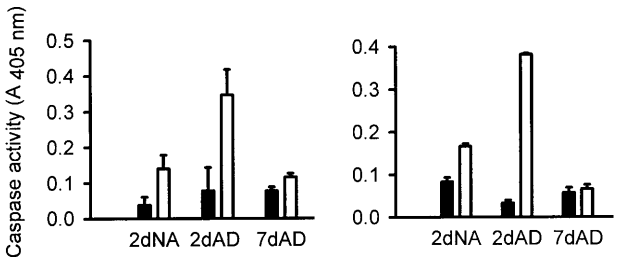

Figure 4 Induction of caspase-9 and caspase-3 activation by removal of M-CSF

After extensive washing, day 2 non-adherent cells (2dNA), day 2 adherent cells (2dAD) and day 7 adherent cells (7dAD) were cultured in the absence of M-CSF for 24 h. Thereafter, cells were washed and lysed for caspase-9 (left panel) and caspase-3 (right panel) assays with specific colorimetric substrates (Leu-Glu-His-Asp-pNA for caspase-9 and Ac-Asp-Glu-Val-Asp-pNA for caspase-3). Results are means ± S.D. for duplicate experiments. Filled columns, M-CSF removed at 0 h; open columns, M-CSF removed at 24 h.

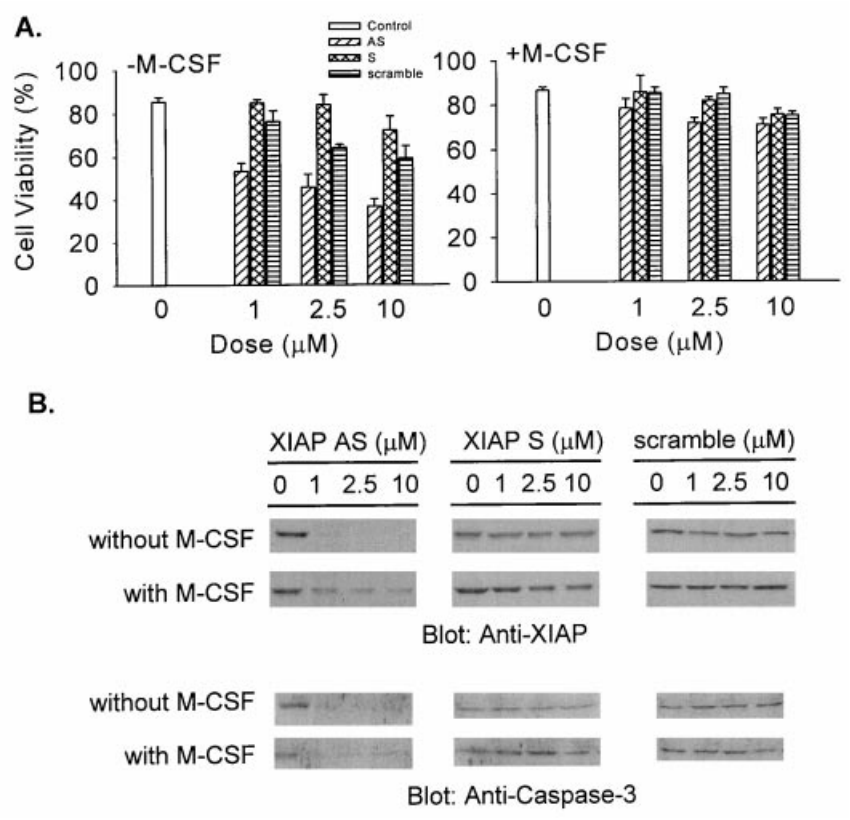

Figure 5 XIAP anti-sense oligos promote M-CSF-depletion-induced apoptosis in day 7 BMDM

(**A**) Day 7 BMDM were treated with various doses of XIAP anti-sense oligos or control oligos [sense (S) and anti-sense (AS) scramble] as indicated in the presence (+) or absence (−) of M-CSF for 24 h. Apoptotic cells were determined by morphological examination after staining with Acridine Orange; 200 cells were scored per sample. Results are means ± S.D. for duplicate experiments. (**B**) Inhibition of XIAP expression by anti-sense oligos. Day 7 BMDM were treated with increasing doses of anti-sense oligos or control oligos for 24 h in the presence or absence of M-CSF. At the end of the treatment, cells were washed and lysed; the total cell lysates were immunoblotted for XIAP. The same blot was washed, stripped and reprobed for caspase-3 with anti-caspase-3 antibodies. Note the degradation of caspase-3 in BMDM treated with anti-sense oligos.

Cytokine depletion-induced apoptosis is associated with the activation of caspase-3 and caspase-9

To understand better the underlying mechanisms of apoptosis, we compared the changes in several apoptosis gene products in day 2 and day 7 BMDM. After extensive washes, day 2 and day 7 BMDM were cultured in the absence of M-CSF for 24–72 h. As shown in Figure 3, the removal of M-CSF from the culture medium resulted in a gradual degradation of XIAP, Bcl-2 and Bax in day 2 BMDM. By 48 h they were all barely detectable. In contrast, no appreciable changes in the levels of XIAP, Bcl-2 and Bax in day 7 BMDM were noticed, even after 72 h of incubation without M-CSF. In addition, the levels of caspase-3 and caspase-9 were decreased in day 2 BMDM, but not day 7 BMDM, after the removal of M-CSF. Prolonged incubation in the absence of M-CSF for 72 h resulted in a complete degradation and loss of both caspase-3 and caspase-9 in day 2 BMDM.

Caspases are activated during apoptosis by multiple proteolytic cleavages of the precursor forms to generate smaller enzymically active complexes [34]. Thus the degradations of caspases shown in Figure 3 suggest that caspase activation had occurred. To confirm that both caspase-3 and caspase-9 were activated in day 2 BMDM by the depletion of M-CSF, we directly measured the activity of caspase-3 and caspase-9 with a colorimetric assay on day 2 non-adherent cells and day 2 BMDM after the removal of M-CSF for 24 h. As expected, both caspase-3 and caspase-9 activities were increased sharply in day 2 non-adherent cells and BMDM, but not in day 7 BMDM (Figure 4), in agreement with results shown in Figure 3.

Promotion of apoptosis in BMDM by XIAP anti-sense oligos

To establish further the role of XIAP in conferring resistance to apoptosis on mature BMDM, we employed an anti-sense approach with 'second-generation' anti-sense oligos that are resistant to serum and cellular nucleases. Treatment of day 7 BMDM with XIAP anti-sense oligos for 20 h abolished their resistance to apoptosis induced by the removal of M-CSF (Figure 5A). At the highest dose of anti-sense oligos (10 μM) used in this study, more than 60% of the day 7 BMDM were induced to undergo apoptosis, in comparison with 20–30% death in cells treated with either sense or anti-sense scramble oligos. The efficacy of anti-sense oligos in decreasing the levels of XIAP in day 7 BMDM was examined by using an immunoblot analysis with anti-XIAP antibody. As shown in Figure 5(B), XIAP anti-sense oligos, but not sense or anti-sense scramble oligos, effectively suppressed the expression of XIAP in the day 7 BMDM. That cell death induced by treatment with anti-sense oligos was mediated through caspase activation was demonstrated by rapid

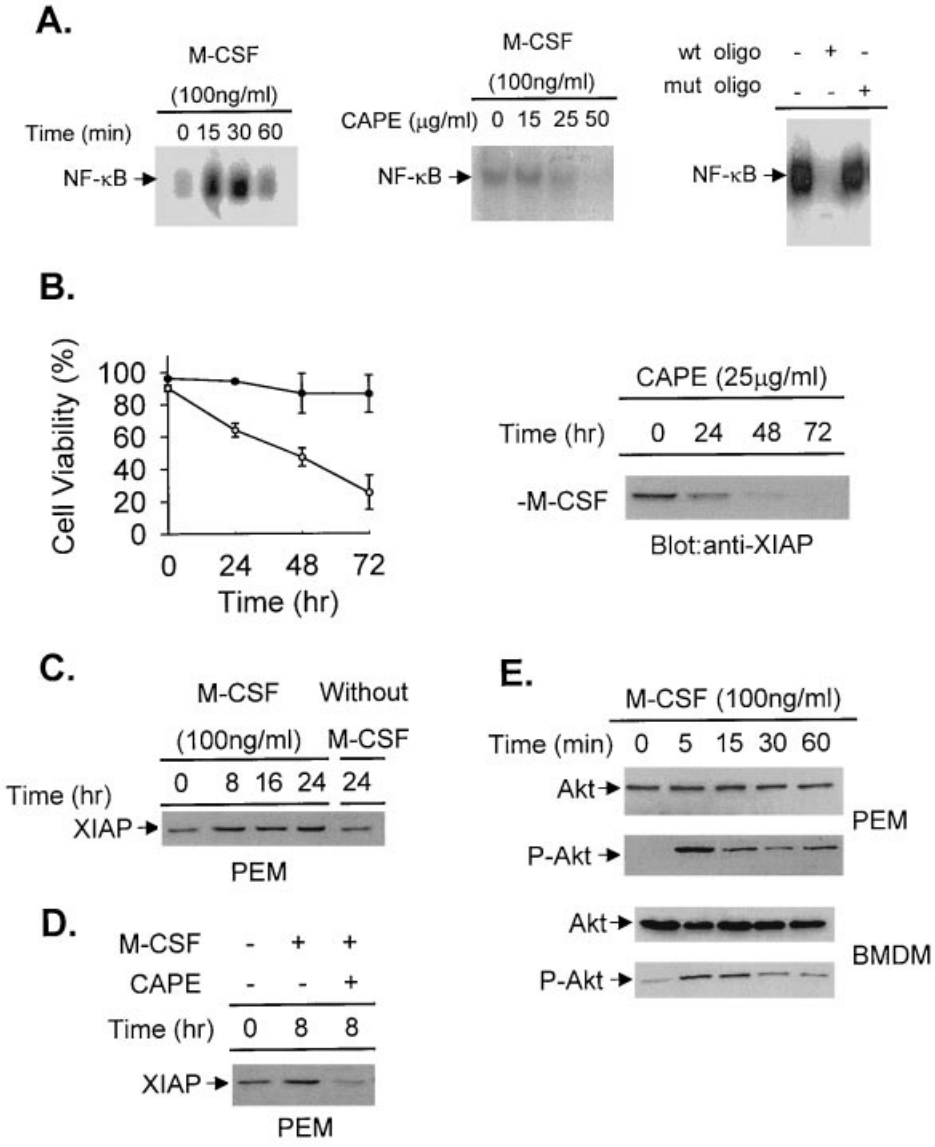

Figure 6 M-CSF up-regulate XIAP expression through activation of NF-κB in BMDM and PEM

(**A**) Effect of M-CSF and CAPE on the activation of NF-κB. After starvation in M-CSF-free medium for 24 h, day 7 BMDM were treated with M-CSF (100 ng/ml) for various periods as indicated or with various doses of CAPE for 24 h. After treatment, cells were lysed and nuclear extracts were isolated, followed by a mobility-shift assay with [γ-^{32}P]ATP-labelled NF-κB consensus sequence (p50) probe. Furthermore, the nuclear extract of day 7 BMDM treated with M-CSF for 15 min was subjected to gel-shift assay including unlabelled wild-type NF-κB p50 oligos or mutant oligos. (**B**) Suppression of XIAP and induction of apoptosis in day 7 BMDM by CAPE. After the removal of exogenous M-CSF, day 7 BMDM were cultured in the presence (○) or absence (●) of CAPE (25 μg/ml) for various periods as indicated. At the end of the incubation period, cell viability was determined after staining with Acridine Orange (left panel). Cell lysates from CAPE-treated samples were further subjected to an immunoblot analysis with anti-XIAP antibody (right panel). Note the disappearance of XIAP in CAPE-treated BMDM. (**C**) Induction of XIAP in PEM cells by M-CSF. After starvation in M-CSF-free medium for 24 h, PEM cells were treated with M-CSF (100 ng/ml) for 0, 8, 16 and 24 h. Total cell lysates were immunoblotted for XIAP. (**D**) Suppression of M-CSF-induced XIAP expression by CAPE. After starvation in M-CSF-free medium for 24 h, PEM cells were treated with or without CAPE for 3 h before the addition of M-CSF (100 ng/ml). After an additional 8 h of treatment, cells were lysed with SDS sample buffer and immunoblotted for XIAP. (**E**) M-CSF induces Akt activation in PEM cells and BMDM. After starvation in medium containing 1% (v/v) FBS for 24 and 12 h respectively, PEM cells and BMDM were treated with M-CSF (100 ng/ml) for 5, 15, 30 and 60 min. Total cell lysates were immunoblotted for Akt and phospho-Akt.

degradations of caspase-3 in day 7 BMDM that had been treated with XIAP anti-sense oligos (Figure 5B).

Up-regulation of XIAP expression by M-CSF is mediated through the activation of NF-κB

The preceding experiments established that the expression of XIAP is associated with macrophage differentiation and resistance to apoptosis in maturity. In the preceding experiment we noted that the addition of M-CSF prevented the decrease in XIAP level in day 7 BMDM that had been treated with anti-sense oligos and decreased apoptosis induced by XIAP anti-sense treatment (Figure 5). These observations led us to hypothesize that M-CSF is involved in the activation of the NF-κB pathway and up-regulation of XIAP during macrophage differentiation. To test this hypothesis, we performed EMSA by incubating 5 μg of

nuclear extracts obtained from M-CSF-treated BMDM with ^{32}P-labelled NF-κB p50 oligos (5′-GCC ATG GGG GGA TCC CCG AAG TCC-3′). As shown in Figure 6(A), NF-κB was indeed activated within 15 min in BMDM on treatment with M-CSF. In another experiment we used CAPE, a specific inhibitor of NF-κB, to block the activation of NF-κB induced by M-CSF. As expected, treatment of day 7 BMDM with CAPE resulted in a marked decrease in the levels of XIAP after the removal of M-CSF. The decrease in XIAP induced by CAPE treatment was accompanied by cell death through an apoptotic mechanism (Figure 6B). The effect of M-CSF in up-regulating the levels of XIAP also was demonstrated in freshly obtained mouse PEM cells, which expressed high levels of XIAP. They were starved in medium containing 5% (v/v) FBS for 24 h, then treated with M-CSF (100 ng/ml) for various periods. As shown in Figure 6(C), the level of XIAP was elevated markedly after stimulation with M-CSF. In contrast, treatment of PEM cells with CAPE (25 μg/ml) for 3 h before the addition of M-CSF completely abolished the up-regulation of XIAP induced by M-CSF (Figure 6D).

A number of recent studies (for example [35]) suggest that the activation of Akt kinase can prevent apoptosis induced by various stimuli. We therefore examined whether Akt activation has a role in conferring resistance on M-CSF-treated cells. The activation of Akt was monitored by immunoblot analysis with monoclonal antibody against active phosphorylated Akt (p-Akt). PEM cells express high levels of Akt proteins but no phosphorylated Akt proteins. Treatment with M-CSF induced a rapid phosphorylation of Akt (5 min) in PEM cells that had been starved in M-CSF-free medium for 24 h (Figure 6E). However, the levels of total Akt proteins were not affect by M-CSF treatment. To confirm that Akt activation had certain effects on M-CSF-induced resistance, we re-added M-CSF (100 ng/ml) into day 7 BMDM cells that had been starved in M-CSF free medium with 1% (v/v) FBS for 12 h. Similarly, a rapid phosphorylation of Akt was detected (5 min) in BMDM after the re-addition of M-CSF (Figure 6E).

DISCUSSION

Using a cell model of BMDM, we showed that immature macrophages and their precursors lack an anti-apoptotic mechanism. They expresses no or very low levels of XIAP, cIAP-1 and Bcl-2, all of which are crucial in protecting the cells from undergoing apoptosis. Expression of these anti-apoptotic regulators is associated with the stage of macrophage differentiation. The levels of XIAP, cIAP-1, Bcl-2 and Bax in BMDM were increased markedly during differentiation, reaching maximum levels after 7–10 days in cultures containing M-CSF, whereas those of caspase-3 and caspase-9 stayed essentially constant. Cell death induced by cytokine depletion in immature cells was mediated through the activation of caspase-9 and caspase-3, both of which are highly expressed in these cells and their precursors. These findings suggest that apoptosis in undifferentiated cells by M-CSF-depletion might be mediated through a cytochrome *c*-dependent pathway, involving the activation of both caspase-9 and caspase-3 in the process [34]. Mature BMDM and tissue-derived macrophages are highly resistant to apoptosis induced by M-CSF depletion. Both cell populations express high levels of caspase-9 and caspase-3, comparable to that detected in bone marrow progenitor cells. However, no activation of either caspase, and hence apoptosis, was observed in these cells after the removal of M-CSF from the culture medium.

We employed an anti-sense approach to investigate further the importance of XIAP in conferring resistance to apoptosis on mature macrophages. Treatment of day 7 BMDM with XIAP anti-sense oligos for 20 h led to a marked down-regulation of XIAP proteins, and promoted apoptosis. The effect of anti-sense oligos in lowering the expression of XIAP is highly specific. Both sense and anti-sense scramble oligos did not lower the levels of XIAP in BMDM. However, treatment with anti-sense oligos cannot completely overcome the resistance to apoptosis in mature macrophages. At the highest concentration (10 μM) used in this study, treatment with anti-sense oligos caused a complete down-regulation of XIAP but induced only approx. 60% of mature BMDM to become apoptotic (Figure 5). This finding led us to speculate that other anti-apoptotic proteins such as cIAP and Bcl-2, both of which were up-regulated during macrophage differentiation, are operative and essential in conferring resistance on differentiated macrophages. Of relevance to our finding is a recent study by Perlman et al. [36] showing that macrophage differentiation is associated with the up-regulation of FLICE-inhibitory protein (Flip), a naturally occurring caspase-inhibitory protein, which confers resistance to Fas-mediated apoptosis.

In the present study, several lines of evidence suggest that the up-regulation of XIAP is under the regulation of NF-κB in M-CSF-treated cells: (1) M-CSF induced an elevated nuclear NF-κB activity in treated BMDM; (2) the activation of NF-κB induced by M-CSF was inhibited by CAPE, a potent NF-κB inhibitor, and (3) inhibition of NF-κB activity resulted in the down-regulation of M-CSF-induced XIAP up-regulation. Further, we showed that M-CSF could partly prevent the down-regulation of XIAP in BMDM treated with anti-sense oligos (Figure 5). The role of NF-κB in conferring resistance to apoptosis also has been reported in other cell models. In a recent study, Hida et al. [37] showed that differentiation of U937 cells induced by PMA is associated with an increased expression of XIAP through the NF-κB pathway. Further, Chu et al. [26] reported that the suppression of tumour necrosis factor-α-induced cell death by cIAP-2 is under NF-κB regulation. These findings indicate that the anti-apoptotic effect of NF-κB is mediated through the up-regulation of IAPs and perhaps other anti-apoptotic proteins in response to various stimuli in these cell models.

The exact mechanism by which M-CSF activates NF-κB is not known. A critical step in the activation of NF-κB is the site-specific phosphorylation of its inhibitor, IκB, by IκB-kinase (IKK) [38]. A recent study showed that Akt is necessary for the activation of NF-κB through the phosphorylation of IKK [39]. In the present study we have shown that M-CSF induced a rapid phosphorylation of Akt before the activation of NF-κB both in mouse tissue-derived macrophages (PEM cells) and in BMDM (Figure 6E), suggesting that the Akt pathway is involved in M-CSF-induced NF-κB activation. Further, by using NIH 3T3 fibroblasts expressing human M-CSF receptors, Kelley et al. [40] showed recently that M-CSF promotes cell survival through the Akt-induced inhibition of caspase-9 activation. Taken together, these findings support the notion that the induction of NF-κB is mediated through the activation of IκB kinase by Akt kinase in M-CSF-treated macrophages.

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