Tumor Necrosis Factor-α-promoted Expression of Bcl-2 and Inhibition of Mitochondrial Cytochrome c Release Mediate Resistance of Mature Dendritic Cells to Melanoma-induced Apoptosis

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Abstract

Melanoma escapes host defenses through a variety of means, including the elimination of immune effector cells within the tumor microenvironment. We have reported recently that murine and human tumors including melanoma induce premature apoptosis of dendritic cells both in vitro and in vivo. In this study, we have demonstrated that overexpression of the Bcl-2 protein family member Bcl-xL rescued murine dendritic cells (DCs) from melanoma-induced death in vitro. Another successful protection approach was tumor necrosis factor (TNF-α)-promoted sustained expression of the antiapoptotic protein Bcl-2 within dendritic cells. This effect of TNF-α was mediated by inhibition of mitochondrial cytochrome c release. Thus, both Bcl-xL and Bcl-2 enhance survival of dendritic cells within the tumor microenvironment. In addition, mature DCs were more resistant to melanoma-induced apoptosis than immature dendritic cells. This finding suggests a stage-dependent sensitivity of DCs to tumor-induced cell death. We conclude that: (a) mature DCs might be more suitable for the use of cancer vaccination; and (b) Bcl-2 protein family members such as Bcl-xL and Bcl-2 rescue DCs from tumor-induced premature apoptosis.

Introduction

DCs are the most potent professional antigen-presenting cells (1). They originate from the bone marrow and migrate into nonlymphoid tissues, where they reside in an immature stage. In vitro, TNF-α promotes DC maturation by up-regulation of adhesion and costimulatory molecules and down-regulation of antigen-capturing and antigen-processing capacity (2). Upon phagocytosis of foreign antigens or activation by TNF-α, DCs undergo functional maturation and migrate to secondary lymphoid organs to initiate specific immune responses in vivo. Ideally, the antigen-presenting DCs should stimulate T cell-mediated antitumor immunity in vivo. Indeed, >25 preclinical studies and several clinical trials have demonstrated the effectiveness of antigen-loaded DCs to mediate antitumor immune responses (3). Therefore, DCs represent one of the most promising tools for experimental immunotherapy for cancer.

However, tumors develop mechanisms to escape recognition by the immune system. Many studies have demonstrated defective immune responses in cancer patients and tumor-bearing animals (4–6). Established tumor-derived immunosuppressive agents include interleukin 10, transforming growth factor-β, vascular endothelial growth factor, gangliosides, NO, and prostaglandin E2. It has been shown recently that tumors, including melanoma, induce apoptosis of T cells by production of Fas ligand (7, 8). However, Fas ligand expression by tumors remains controversial (9) and appears to be stage dependent (10). DCs have been demonstrated to represent another target for tumor (11). Virtually every tumor including melanoma induces premature apoptosis of both murine and human DCs (12–15). Subtypes such as monocyte-derived and CD34+ precursor-derived DCs are equally sensitive (12). This finding has been confirmed recently by others for monocyte-derived DCs (16). Tumor-induced DC apoptosis does not only occur in vitro but also in vivo (12). In addition, tumors have been shown to exhibit a systemic rather than a local effect on DC function (17). Therefore, tumor-induced apoptosis of DCs is likely to represent another strategy of neoplasms to escape from immune recognition and elimination.

The aim of this study was to compare the sensitivity of mature and immature DCs to melanoma-induced apoptosis and to evaluate mechanisms involved in the regulation of DC resistance to melanoma-induced cell death. We showed that TNF-α-promoted sustained expression of Bcl-2 protects DCs from melanoma-induced apoptosis. This effect appeared to be attributable to TNF-α-mediated inhibition of mitochondrial cytochrome c release within DCs. Thus, melanoma-induced DC death is regulated, and can be prevented, by antiapoptotic proteins. In addition, maturation of DCs enhanced their survival within the melanoma microenvironment. We also demonstrated in this study that overexpression of the Bcl-2 family member Bcl-xL rescues DCs from B16 melanoma-induced apoptosis in vitro. This result suggests another approach for protecting DCs in the tumor microenvironment.
Materials and Methods

Mice. Male C57BL/6 mice, 4–6 weeks of age, were obtained from Taconic (Germantown, NY) and housed in transparent plastic cages at constant temperature (20 ± 1°C) and humidity (50 ± 5%). Animals had access to food and water ad libitum and were aclimatized for at least 2 weeks prior to experimental manipulations. All experimental procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Tumor Cell Cultures. The murine B16 melanoma cell line was cultured in complete RPMI 1640 (Life Technologies, Inc., Grant Island, NY) supplemented with 10% heat-inactivated fetal bovine serum. Tumor cells were routinely tested for the common species of Mycoplasma by enzyme-linked immunosassay (Boehringer Mannheim, Indianapolis, IN).

DC Generation, Maturation, and Coincubation with Effector Cells. Murine DCs were generated as described previously (14). Briefly, bone marrow cells were first depleted of erythrocytes. Incubation with antibodies [partially purified supernatants of hybridoma cell cultures TIB-146, TIB-207, and TIB-105 (American Type Culture Collection) for anti-B220, anti-CD4, and anti-CD8 antibodies, respectively] for 1 h at 4°C and rabbit complement for 30 min at 37°C depleted B and T lymphocytes. Overnight culture was performed at a concentration of 10⁶ cells/ml at 37°C in complete medium supplemented with 10% fetal bovine serum, 1 µg/ml indomethacin, and 50 µM N-methyl-L-arginine in six-well plates (Falcon, Franklin Lakes, NJ). Nonadherent cells were resuspended at a concentration of 2.5 × 10⁵ cells/ml at 37°C in complete medium supplemented with recombinant murine granulocyte/macrophage-colony-stimulating factor (1000 units/ml) and recombinant murine interleukin 4 (1000 units/ml). Cells were cultured in six-well plates at 4°C. Distilled water (Sigma) and 10 µg/ml leupeptin (Sigma). The homogenate was centrifuged at 10,000 g for 30 min at 4°C and the resulting supernatant was harvested 5 days after transfection. To determine the level of Bcl-xL, DCs were harvested 5 days after transfection. To determine the level of Bcl-2 or cytochrome c, DCs were harvested 48 h after initiation of coincubation with B16 melanoma cells. DCs were washed twice with PBS (pH 7.4) and lysed in 100 µl of ice-cold distilled H₂O containing 1% Triton X-100 and a mixture of antiproteases consisting of 0.5 mM phenylmethylsulfonyl fluoride (Sigma) and 10 µg/ml leupeptin (Sigma). The homogenate was centrifuged at 12,000 × g for 15 min at 4°C. Protein concentration in the supernatant was determined by Bradford with Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Samples were boiled, and 10 µg of protein were loaded per lane on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane (Amersham Life Science, Piscataway, NJ). The membrane was blocked in a solution containing 5% powdered nonfat milk in TTBS buffer (150 mM NaCl, 0.05% Tween 20, and 10 mM Tris-HCl, pH 7.6) at 4°C overnight, and probed with rabbit primary antibodies against Bcl-xL (Oncogene Research Products, Boston, MA), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), or cytochrome c (PharMingen, San Diego, CA). Membranes were washed twice and treated with donkey antirabbit secondary antibody (Amersham). Bound antibodies were visualized using chemiluminescence reagent according to the manufacturer’s protocol (DuPont NEN). The bands obtained were analyzed using UN-SCAN-IT gel software (Silk Scientific, Inc., Orem, Utah). Pixel density was determined for each lane after background subtraction. Results were expressed as relative percentages.

Quantification of DNA Fragmentation. DNA fragmentation was determined using the JAM assay (19) with minor modifications (12). DNA of cultured day 6 DCs was radiolabeled for 24 h using 3 µCi/ml [³H]thymidine (DuPont NEN, Boston, MA). Dead cells were removed by NycoPrep cell separation (Nycomed Pharma AS, Oslo, Norway), resulting in the viability >99%. Radiolabeled target cells (1 × 10⁶) were added in triplicates to B16 melanoma cells at E:T ratios up to 14:1. Round-bottomed 96-well plates with a final cell suspension volume of 200 µl/well were used. Eight h later, cells were harvested onto GF/C glass fiber filter using a Mach III harvester (Tomtec, Hamden, CT). The radioactivity of intact chromosomal DNA was retained on each filter and determined by ³-scintillation counting using a 1450 MicroBeta Trilux Counter (Wallac, Gaithersburg, MD). The percentage of DNA fragmentation was calculated using the following equation: % DNA fragmentation = (cpmcontrol − cpmexperimental)/cpmcontrol × 100, where cpmcontrol is cpm of retained DNA in the absence of B16 melanoma cells, and cpmcontrol is cpm of retained DNA in the absence of B16 melanoma cells.

Preparation of Mitochondrial Fraction. DCs were harvested in isosotic mitochondrial buffer (20 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 mg/ml aprotinin), and homogenized with a Dounce by 15–20 strokes. Samples were transferred to Eppendorf tubes and centrifuged at 500 × g for 5 min at 4°C to eliminate nuclei and unbroken cells. The resulting supernatant was centrifuged at 10,000 × g for 30 min at 4°C. Further centrifugation at 100,000 × g for 1 h at 4°C yielded the final soluble cytosolic fraction, S100, which was assessed for the presence of cytochrome c.
Results

**TNF-α Enhanced DC Viability during Coincubation with Melanoma Cells.** To determine whether DC survival within the tumor microenvironment depends on the stage of DC maturation, we evaluated the effect of TNF-α on the sensitivity of murine DCs to melanoma-induced apoptosis. DC viability was determined 48 h after the initiation of DC coincubation with either B16 melanoma cells or splenocytes as a control. Approximately 400 TNF-α-treated and nontreated DCs were examined in each group using trypan blue exclusion analysis. We found that TNF-α-promoted maturation of DCs significantly abrogated tumor-induced DC death (Fig. 1). For instance, immature DCs exhibited death rates of 25 ± 3% after coincubation with B16 melanoma cells for 24 h, whereas mature DCs demonstrated death rates of only 10 ± 2% (P < 0.01). Thus, TNF-α-induced maturation of DCs results in enhanced resistance to melanoma-induced death.

**TNF-α Abrogated Melanoma-induced DNA Fragmentation in DCs.** To confirm the effect of TNF-α on DC viability, we next evaluated the ability of TNF-α to prevent melanoma-induced DNA fragmentation in murine DCs in vitro. JAM assays were performed as described above. Four independent experiments were carried out, and representative data are shown in Fig. 2. As shown, pretreatment of DCs with TNF-α protected DCs in particular at high E:T ratios. For example, at an E:T ratio of 14:1, the level of DNA fragmentation in immature DCs were 60 ± 5%, whereas mature DCs exhibited a death rate of only 40 ± 3% (P < 0.01). Thus, these data suggest that maturation of DCs in cultures is accompanied by increased resistance to melanoma-induced apoptotic death.

**TNF-α Abrogated Melanoma-induced Inhibition of Bcl-2 Expression within DCs.** The aim of the next series of experiments was to evaluate whether the antiapoptotic effect of TNF-α is mediated by Bcl-2 protein within DCs. Western blot analysis revealed B16 melanoma-induced inhibition of Bcl-2 expression in DCs: 36.5% versus 0.02% relative pixel intensity in treated and control cell extracts, respectively, P < 0.01 (Fig. 3). This inhibitory effect of tumor cells was completely abrogated by pretreatment of DCs with TNF-α; the relative pixel intensities of protein bands were 32.5 and 31% in B16 melanoma-treated and nontreated DCs, respectively (P > 0.05). These data thus suggest the involvement of Bcl-2 protein family members in TNF-α-induced enhanced survival of murine DCs within the melanoma microenvironment.
TNF-α Abrogated Melanoma-induced Mitochondrial Cytochrome c Release in DCs. Bcl-2 is capable of preventing apoptosis by inhibition of cytochrome c release (20, 21). We therefore evaluated whether TNF-α-promoted sustained expression of Bcl-2 in DCs results in inhibition of mitochondrial cytochrome c release in these cells. The results of Western blotting analysis of cytochrome c in treated and nontreated DCs revealed the ability of TNF-α to prevent B16 melanoma-induced release of cytochrome c in DCs (Fig. 4). In fact, TNF-α pretreatment of DCs significantly decreased detected cytochrome c levels of cytochrome c release was also observed in control DC cultures, which were not coincubated with tumor cells: 24.1% versus 7.9% in TNF-α nontreated and treated cultures, respectively. Taken together, these results suggest that: (a) mitochondrial apoptosis-related proteins are involved in the regulation of melanoma-induced death of DCs; and (b) their expression is differentially regulated during DC maturation in cultures.

Overexpression of Bcl-xL Protected DCs from Melanoma-induced Apoptosis. To evaluate whether other Bcl-2 family members also would enhance DC survival within the melanoma microenvironment, we transfected murine DCs with the gene encoding murine Bcl-xL using an adenoviral vector (18). Western blot analysis consistently revealed high levels of Bcl-xL protein expression 1 week after transfection (data not shown). In addition, evaluation of long-term DC cultures showed that overexpression of Bcl-xL in DCs significantly increased the survival of terminally mature DCs (P < 0.01; data not shown). We therefore performed [3H]DNA fragmentation (JAM) assays to compare the survival of transfected and control DCs in the presence of melanoma cells. The analysis of these results demonstrated that Bcl-xL transfection of DCs resulted in a significantly decreased level of B16 melanoma-induced apoptosis from 39.7 ± 4.6% to 17.2 ± 3.1% (P < 0.01; Fig. 5). Thus, overexpression of antiapoptotic proteins represents a potential approach of improving the outcome of DC-based clinical cancer trials.

Discussion

Apoptosis was initially defined as shrinkage and fragmentation of both cell cytoplasm and nucleus accompanied by degradation of chromosomal DNA (22). More recently, it was found that cystein proteases, present in almost all nucleated animal cells and known as caspases, represent a key regulator of programmed cell death (23). However, apoptosis can also occur without activation of caspases. In fact, Bax-induced alterations in mitochondrial function and subsequent cell death do not necessarily require activation of caspases (24). Although Kerr et al. (22) originally noted that the ultrastructure of cytosolic organelles including mitochondria appeared to be well preserved within apoptotic cells, mitochondria were later identified as a key regulator of programmed cell death (25). The first evidence for the involvement of a mitochondrial protein in the initiation of apoptosis was the finding that cytochrome c represents an essential component of the complex activating the death protease caspase 3 (CPP32; Ref. 26). Confocal microscopy revealed that mitochondria release their cytochrome c within 5 min after stimulation (27). This process is complete and kinetically invariant. However, the precise mechanism is still the subject of debate.

Cytochrome c translocation from mitochondria to the cytosol is regulated by the Bcl-2 family of proteins. Bcl-2 (20, 21) and Bcl-xL (28) inhibit apoptosis by preventing cytochrome c release, whereas Bax (29) and Bak (30) promote its release from mitochondria. Bcl-2 is located predominantly in the mitochondrial outer membrane and, to a lesser degree, inner membrane, as well as in the endoplasmic reticulum and in the nuclear membrane (31). In contrast, cytochrome c is located on the outside of the inner mitochondrial membrane. The mechanism of interaction between Bcl-2 and cytochrome c still awaits elucidation. Liberated cytochrome c is required for the association of caspase-9 and apoptotic protease activating factor-1 (32). This event results in caspase-9 activation. Activated caspase-9 in turn cleaves and activates caspase-3. However, the functional hierarchy among these proteins is not defined yet.

We have demonstrated recently that murine and human tumors including melanoma induce premature apoptosis of DCs (12, 13, 14, 15). The aim of this study was to evaluate the sensitivity of immature and mature DCs to melanoma-induced cell death. We determined the ability of Bcl-2 protein family
members to enhance DC survival within the tumor microenvironment by transfection of murine DCs with the gene encoding murine Bcl-xL. Overexpression of Bcl-xL protein in DCs resulted in enhanced survival of DCs in long-term culture. We next performed [3H]DNA fragmentation assays to evaluate the effect of Bcl-xL overexpression on DC survival within the melanoma microenvironment. Bcl-xL transfection of DCs significantly decreased the level of B16 melanoma-induced DC apoptosis (Fig. 5). This finding reveals the involvement of Bcl-xL in the regulation of tumor-induced DC death and also suggests a potential approach of enhancing DC survival within the tumor microenvironment.

Because members of the Bcl-2 family of proteins are inducible by TNF (33), we next evaluated the potential protective effect of TNF-α on tumor-induced DC death. Coculture experiments revealed that TNF-α-induced maturation of DCs protected them from B16 melanoma-induced cell death (Fig. 1). In addition, JAM assays consistently revealed lower levels of melanoma-induced DNA fragmentation in mature DCs (Fig. 2). These results suggest that mature DCs are more resistant to melanoma-induced apoptosis. Thus, the tumor seems to attack DCs specifically in their early developmental stage. As expected, direct contact between effector and target cells resulted in pronounced DC death rates in JAM assays. In contrast, trypan blue exclusion experiments demonstrated lower DC death rates because of the separation of effectors and targets through inserts. In conclusion, the use of mature instead of immature DCs for cancer vaccination might result in better clinical responses because high numbers of DCs within the tumor microenvironment are associated with a more favorable prognosis (11, 34).

We next evaluated whether the antiapoptotic protein Bcl-2 mediates the protective effect of TNF-α on the survival of tumor-associated DCs. Bcl-2 prevents most forms of apoptotic cell death as well as certain forms of necrotic cell death, suggesting that apoptosis and some forms of necrosis share particular steps (35). The bcl-2 (B-cell leukemia/lymphoma 2) gene was originally described as a proto-oncogene at the breakpoint of a t(14;18) chromosomal translocation associated with follicular B-cell lymphoma (36, 37). Later, Bcl-2 was identified as the first antiapoptotic protein (38). We therefore evaluated whether TNF-α renders DCs more resistant to melanoma-induced apoptosis by preventing down-regulation of Bcl-2 expression in DCs. Western blot data analysis suggests the ability of TNF-α to prevent B16 melanoma-induced down-regulation of Bcl-2 expression in DCs (Fig. 2). This result demonstrates a potential therapeutic approach of improving DC-based immunotherapy by overexpression of Bcl-2 within DCs to abrogate tumor-induced DC death. In conclusion, therapeutic overexpression of antiapoptotic proteins within DCs represents a testable approach for improving immunotherapy of cancer including melanoma. In fact, adenoviral transfection of murine DCs with the gene encoding Bcl-xL has been demonstrated to improve the therapeutic outcome of cancer vaccination in a mouse model (18).

Antiapoptotic proteins are capable of preventing mitochondrial cytochrome c release. This dependence has been shown for both Bcl-xL (28) and Bcl-2 (20, 21). We therefore evaluated whether TNF-α-promoted sustained expression of Bcl-2 prevents melanoma-induced cytochrome c release in murine DCs. We isolated the mitochondrial fraction of murine DCs after coincubation with B16 melanoma cells. Western blot analysis revealed the ability of TNF-α to abrogate melanoma-induced mitochondrial cytochrome c release within DCs (Fig. 4). This finding suggests the involvement of cytochrome c in the regulation of TNF-α-mediated enhancement of DC survival within the tumor microenvironment.

In summary, we have demonstrated that TNF-α enhances resistance of DCs to melanoma-induced apoptosis. Because CD154 also protects DCs within the tumor microenvironment (14), we can conclude that the survival of tumor-associated DCs appears to depend on their stage of maturation. Tumors seem to escape immune recognition in part by preventing immature DCs from obtaining access to tumor antigens. The specific sensitivity of immature DCs to melanoma-induced apoptosis suggests the use of mature DCs for cancer vaccination. However, DCs need to take up antigens prior to T-cell stimulation. Therefore, overexpression of antiapoptotic proteins such as Bcl-2 could represent a more successful approach of enhancing the survival of functional tumor-associated DCs compared with simply using more mature DCs for immunotherapy. This notion is supported by the results of a successful study using Bcl-xL-transfected DCs for cancer vaccination in mice (18). Our finding that TNF-α-promoted sustained expression of Bcl-2 is accompanied by inhibition of mitochondrial cytochrome c release within DCs identifies cytochrome c as another player involved in the regulation of melanoma-induced DC death. However, mitochondria are not the only regulators of apoptotic death. Cell lines lacking mitochondrial DNA can still be induced to die by apoptosis (39).

In addition, those cells can be protected from apoptosis by the overexpression of bcl-2 (39). Thus, neither apoptosis nor the protective effect of bcl-2 necessarily depend on functional mitochondria. In conclusion, further studies are necessary to identify additional mechanisms of melanoma-induced DC death. A complete picture of the functional hierarchy of all players involved in the regulation of tumor-induced DC apoptosis will allow us to define the most effective means of enhancing the survival of tumor-associated DCs. This approach will improve the outcome of DC-based immunotherapy for cancer.

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Clin Cancer Res 2001;7:974s-979s.