Stimulation of CD40 on Immunogenic Human Malignant Melanomas Augments Their Cytotoxic T Lymphocyte-mediated Lysis and Induces Apoptosis

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INTRODUCTION

CD40, a Mr 45,000–50,000 integral membrane protein, is a member of a protein family that includes the TNF-R1,3 TNF-RII, p75 nerve growth factor receptor, CD30, CD27, Fas, and others (reviewed in Refs. 1 and 2). Expression and function of CD40 on B cells have been studied intensively (reviewed in Refs. 1 and 2). Several reports show that the expression of CD40 is not restricted to cells of the B lineage because CD40 is functionally expressed on dendritic cells, monocytes, vascular endothelial cells, keratinocytes, and fibroblasts (reviewed in Refs. 1–3). The ligand for CD40 (CD40L, CD154, TRAP, or gp39), a type II membrane protein with significant sequence homology to TNF, is primarily expressed on activated CD4+ T cells but is also found on stimulated CD8+ T cells, mast cells, eosinophils, B cells, and dendritic cells (2, 4). CD40 ligation induces functional changes in hematopoietic and nonhematopoietic cells, including the up-regulation of MHC, costimulatory, and adhesion molecules and the secretion of cytokines, which all participate in T cell-mediated immune responses (reviewed in Refs. 1–3). Furthermore, a CD40 stimulus is an important factor for the induction of CTLs (4–9).

However, consequences of CD40 stimulation are not limited to the induction of T cell- or B cell-mediated immune responses because cross-linking of CD40 has also profound effects on cell proliferation and viability. Ligation of CD40 results in enhanced proliferation of fibroblasts and B cells (reviewed in Refs. 1 and 2), but inhibition of cell proliferation is described for keratinocytes and neoplastic cells such as B lymphomas (reviewed in Refs. 1–3). Moreover, following CD40 signaling apoptosis is induced in the human myeloma cell line XG2 (10) and in transformed cells of mesenchymal and epithelial origin (11).

MM has been described as an immunogenic tumor, which is infiltrated by CD4+ and CD8+ T cells (12). MM cells have been shown to express both MHC class I and II molecules and to stimulate the proliferation of autologous CD4+ and CD8+ T lymphocytes in vitro (13). Furthermore, human MM cells are susceptible to specific lysis by CD8+ CTLs that recognize MHC class I-associated peptides derived from different melanoma-specific antigens (reviewed in Ref. 14). Additionally, human MM can produce a range of cytokines capable of enhancing T cell-mediated immunity, including IL-1, TNF-α, IL-6, IL-8, and GM-CSF (15). However, the mechanisms by which cytokine expression of MM is regulated are largely unknown. A potent transcription factor of these cytokines is NF-κB, which is known to be activated, among other stimuli, by CD40 triggering on hematopoietic and nonhematopoietic cells (reviewed in Ref. 16).

Recently, it has been described that MM cells express CD40 both in vivo and in vitro (17, 18), but little is known about its function. Here, we demonstrate in some CD40-positive immunogenic human MM that CD40/CD40L-mediated signals activate transcription factor NF-κB, enhance the production of proinflammatory cytokines as well as of immunorelevant surface molecules, and augment their specific CTL-mediated lysis. Moreover, CD40 triggering markedly inhibits the growth of these MM cell lines via the induction of apoptosis.

MATERIALS AND METHODS

mAbs and Reagents. mAb with specificity for the following human antigens were used: CD40 (EA-5; Calbiochem, Bad Soden, Germany); CD44 (MEM-85; Monosan, Uden, The Netherlands); CD45 (84H10; Immunotech, Hamburg, Germany); CD40L (TRAP); anti-HLA-ABC (W6/32; Sigma, Deisenhofen, Germany); and anti-HLA-DR (Tu36), B7.1 (BB1), B7.2 (IT2.2), CD95 (DX2), CD95L (NOK-1), and antimouse H2-Kb mAb (36-7.5), all purchased from PharMingen (Hamburg, Germany). The anti-CD40 mAb (G28-5), anti-CD40L mAb (39-1.106), and soluble CD40L fusion protein (scCD40L), consisting of the extracellular domain of mouse CD8 and the extracellular domain of human CD40L, were described previously (19). The fusion protein was used in a 1:5 dilution of supernatants collected from transfected COS cells unless stated otherwise. Isotype-matched control mAbs were purchased from Immunotech. The following recombinant human cytokines, receptors, and fusion proteins were used: IL-2, IFN-γ, and IL-1β (PromoCell, Heidelberg, and Genzyme, Rüsselsheim, Germany), TNF-α and sTNFRI (R&D Systems, Wiesbaden, Germany), AP01/Fas-immunoglobulin (Alexis, Grünberg, Germany).

Cell Lines, Media, and Cell Culture. The human melanoma cell lines MRW, MM1, HT144, and SK-Mel28 were purchased from American Type
Culture Collection (Manassas, VA); MV3 and IF6 were a gift from Dr. G. P. van Muijen (Department of Pathology, University of Nijmegen, Nijmegen, The Netherlands); Term 1–3 were generated in our laboratory from lymph node melanoma metastases, 384/33 and 397/22, which were generated from spontaneously regressing MM and were a gift from Dr. A. Mackensen (Department of Medicine I, University of Freiburg, Freiburg, Germany); and LB39-Mel, LB373, LB265, LB33-Mel, LB24, LG2-Mel, and M2Z-Mel.43 and the MelanA/MART-1–specific CTL clone LB373-CTL-246/15 were established at the Ludwig Institute for Cancer Research (Brussels, Belgium). The Daudi cell line and the fibroblast cell line L-M from CSH mice (the CD40L-transfected and the untransfected control cell line) were obtained from American Type Culture Collection (Manassas, VA); Termel 1–3 were generated in our laboratory from lymph node melanoma metastases of melanoma cells, with or without IFN-α, 50 μg/ml penicillin-streptomycin, and 2 mm l-glutamine (all from Life Technologies, Inc.). Cultures of nontransformed melanocytes were established from human neonatal foreskin by digestion with dispase (2 h at 37°C) and trypsin (10 min at 37°C) and maintained in selective melanocyte growth medium (PromoCell) in a humidified atmosphere at 5% CO2. Second- to fifth-passage melanocyte cultures were used for all experiments. MM cells and murine L cells were cultured at 37°C in a humidified atmosphere at 5% and 8% CO2, respectively. CTL clone LB373-CTL-246/15, recognizing the MelanA/MART-1 peptide 27–35 (EAAAGGILTV), was maintained in culture as described (21). In brief, 2 × 10^5 CFTLs were transferred every 7 days to 2 ml of Iscove’s modified DMEM containing 10% human serum and 50 units/ml IL-2, 1 × 10^6 T2 cells (α-irradiated, 100 Gy) pulsed with 1 μM peptide, and 1.5 × 10^7 EBV-transformed feeder B cells (γ-irradiated, 100 Gy). Immunohistochemistry. After the patients gave their informed consent, we obtained punch biopsies at the time of surgery from tumors of patients with metastatic melanoma. Frozen sections of melanoma tissue were exposed to primary antibody and then washed with medium. T2 cells were either pulsed with MelanA/MART1 peptide, and 5 × 10^6 EBV-transformed feeder B cells (<i>γ</i>-irradiated, 100 Gy). Alternatively, equal numbers of melanoma cells, with or without IFN-γ pretreatment, were cultured on a monolayer of mitomycin C-treated (50 μg/ml, 30 min at 25°C) CD40L- L-cells or control L-cells for 48 h at 37°C/5% CO2. The cells were detached with 2 ml EDTA/PBS, and L cells were removed by immunomagnetic separation after incubation with a H2-K<sup>b</sup>-specific mAb using the Dynabeads system (Dynal, Hamburg, Germany).

**Cytokine ELISA.** Cell culture supernatants were assayed for the following human cytokines by specific ELISA according to the manufacturer’s instructions: IL-1α, IL-6, IL-10, transforming growth factor β1, basic fibroblast growth factor, TNF-α, and GM-CSF (all from R&D Systems). Data are expressed as mean values of triplicate measurements ± SD.

**EMSA.** EMSA was performed as described (22). In brief, total cell extracts were prepared using a high-salt detergent buffer (Toxo; 20 mm Heps (pH 7.9), 350 mm NaCl, 20% (w/v) glycerol, 1% (w/v) NP40, 1 mg MgCl<sub>2</sub>, 0.5 mm EDTA, 0.1 mm EDTA, 0.5 mm DTT, 0.1% PMSF, and 1% apotinin). Cells were harvested by centrifugation, washed once in ice-cold PBS (Sigma) and resuspended in four cell volumes of Toxo buffer. After 30 min on ice, the lysates were centrifuged for 5 min at 13,000 × g at 4°C. The protein content of the supernatant was determined and equal amounts of protein (10–20 μg) added to a reaction mixture containing 20 μg of BSA (Sigma), 2 μg of polyclonal antibody (Boehringer-Mannheim, Germany), 2 μl of buffer D+ (20 mm Heps (pH 7.9), 20% glycerol, 100 mm KCl, 0.5 mm EDTA, 0.25% NP40, 2 mm DTT, and 0.1% PMSF), 4 μl of buffer F (20% Ficoll 400, 100 mm Heps, 300 mm KCl, 10 mm DTT, and 0.1% PMSF), and 100,000 cpm (Cerenkov) of a 32<sup>P</sup>-labeled oligonucleotide in a final volume of 20 μl. Samples were incubated at room temperature for 25 min. NF-κB oligonucleotide (Promega, Mannheim, Germany) was labeled using (γ-32<sup>P</sup>)ATP (3000 Ci/mmole; Amer sham) and T4 polynucleotide kinase (Promega) as described (23).

**Proliferation Assay.** MM cells at 10 × 10<sup>5</sup>, 5 × 10<sup>5</sup>, and 2 × 10<sup>5</sup> were cultured on monolayers of 2 × 10<sup>4</sup> CD40L-transfected or untransfected mitomycin C-treated L-cells in 96-well flat-bottomed plates (Becton Dickin son) for 36 h. In some experiments, either biologically active sTNF-R1 (0.5 μg/ml) or APO1/Fas-immunoglobulin (20 μg/ml) to neutralize MM derived TNF-α or APO1/FasL was added to the coculture. [3H]Thymidine (Amersham, Braunschweig, Germany) was added at 1 μCi/well for the last 18 h of culture. Plates were harvested with a Canbrera Packard Filter-Mate (Canberra Packard, Dreieich, Germany) and incorporation of [3H]thymidine was determined by liquid scintillation spectroscopy using a TopCount device (Canberra Packard). Data are expressed as mean cpm ± SD of quadruple cultures.

**Cytotoxicity Assay.** 51Cr-release assays, performed as described (13), were used to assess cytotoxicity rates of the CTL clone LB373-CTL-246/15 (specific for MelanA/MART1 peptide 27–35) against the MM cell line LB39-Mel (HLA-A2<sup>-</sup>, MelanA/MART1<sup>+</sup>) or against the HLA-A2<sup>-</sup> TAP-deficient T-B lymphoblast line T2. LB39-Mel cells with or without IFN-γ priming (200 units/ml, 24 h) were cocultured with myeloma-tumor C-treated L-cells (CD40L-transfected or control) and then purified by immunomagnetic separation as described above. Thereafter, target cells (either LB39-Mel or T2) were labeled with 1 μCi/ml Na<sub>2</sub>CrO<sub>4</sub> (Amersham) for 1 h at 37°C/5% CO2 and then washed with medium. T2 cells or other pertinent tumor cells were labeled with EAAAGGILTV at 1 μM immediately prior to use (positive control) or were left untreated (negative control). Cr<sup>51</sup>-labeled target cells (2 × 10<sup>5</sup> cells/well) were incubated with LB373-CTL-246/15 at the indicated E:T ratios for 4 h at 37°C in a final volume of 200 μl in V-bottomed 96-well plates (Becton Dickinson). Spontaneous 51Cr-release was determined by culture of identical numbers of labeled target cells without CTLs; maximum 51Cr-release by addition of 1% Triton X-100/PBS to target cells. Fifty μl per well were then transferred onto Luma plates (Canberra Packard) dried and analyzed using a TopCount device. Percentage of specific lysis was calculated according to the formula:

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\% \text{ specific lysis} = \left( \frac{\text{test } \text{51 Cr release} - \text{spontaneous } \text{51 Cr release}}{\text{maximum } \text{51 Cr release} - \text{spontaneous } \text{51 Cr release}} \right) \times 100
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Triton X-100). DNA was extracted by incubation of cell lysates with ice-cold TTE buffer, 2-propanol, and 5 M NaCl (5:7:1) at 4°C overnight. Lysates were then centrifuged at 14,000 rpm for 30 min, and the DNA-containing pellet was air-dried for 3 h at RT. After solubilization of the pellet with TE buffer, DNA samples were separated in 2% agarose/Tris-borate-EDTA gels. Afterward, DNA was stained with ethidium bromide, and gels were photographed using a Herolab E.A.S.Y RH3 device. Alternatively, detection of DNA fragmentation was performed using the terminal deoxynucleotidyltransferase-mediated dUTP-X nick end labeling assay. In brief, 10⁶ MM cells were stained with the in situ cell death detection kit from Boehringer (Mannheim, Germany) according to the manufacturer’s instructions and subsequently analyzed on a FACScan using CellQuest software (Becton Dickinson).

RESULTS

CD40 Expression by Human Melanoma Cells in Situ and by Melanoma Cell Lines in Vitro. Immunohistology of cryosections from tumor specimen of MM precursor lesions as well as of established MM revealed CD40 expression by the tumor cells of premalignant dysplastic nevi; however, no CD40 could be detected on normal epidermal melanocytes (Fig. 1A). We observed strong surface expression of CD40 on melanoma cells within all specimens of primary tumors of melanoma (Fig. 1B). In contrast, in MM metastases, CD40 staining on the tumor cells was weak or absent, whereas the
endothelial cells of intratumoral vessels retained their strong CD40 surface expression (Fig. 1C). In aggregate, we observed a gradual loss of CD40 surface antigen during melanoma progression.

To confirm and extend these results, cultured human MM cell lines were examined for surface expression of CD40 by flow cytometry. Staining of melanoma cell lines with the CD40-specific antibodies G28.5 and EA-5 revealed 7 of 18 to be CD40 positive. Fig. 1D shows three representative cell lines positive for the CD40 antigen. Interestingly, 2 of 7 CD40-positive MM cell lines were established from highly immunogenic melanomas, whereas the other CD40 positive MM cell lines were established from MM metastases, characterized by slow local tumor growth and low metastatic potential in nu/nu mice (23). On the other hand, all CD40-negative MM cell lines were established from rapidly growing advanced stages of melanoma metastases.

IFN-γ was shown to up-regulate CD40 expression on cultured human keratinocytes, thymic epithelial cells, and vascular endothelial cells, whereas TNF-α and IL-1β had no effect (reviewed in Refs. 1–3). To investigate whether these cytokines modulate the level of CD40 on MM, flow cytometry was performed after IFN-γ, TNF-α, IL-1β, and CD40 stimulation. A 48-h treatment with IFN-γ (200 units/ml) or TNF-α (50 ng/ml) led to a 2-fold increase in the MFI of CD40 surface staining, whereas IL-1β (1 μg/ml; Fig. 2, A and D) and CD40 triggering (CD40L+L-cells or sCD40L) had no effect (Table 1 and data not shown). Of note, none of these treatments could induce CD40 expression in CD40 negative MM cell lines (data not shown). A weak CD40 surface expression was also observed on 3 of 3 human melanocyte cell lines cultured in melanocyte growth medium containing insulin and phorbol 12-myristate 13-acetate (data not shown).

CD40 mRNA levels were determined in human MM by Northern blot analysis. A single 1.3–1.4-kb mRNA transcript was observed in the MM cell line MIRW (Fig. 1E), corresponding to the CD40 mRNA transcript described for human B cells and the MM cell line HS294T (24). Stimulation of MIRW with IFN-γ (200 units/ml) or TNF-α (50 ng/ml) resulted in a 2–3-fold induction of CD40 transcription levels as early as 8 h after cytokine addition (Fig. 1E). In contrast, IL-1β had no effect (Fig. 1E). Again, neither IFN-γ nor TNF-α induced CD40 RNA transcription in MM that did not constitutively express CD40 antigen (data not shown). These data indicate that the IFN-γ and TNF-α induced up-regulation of surface CD40 expression (Table 1) is likely to be the result of increased CD40 mRNA transcription.

CD40 Stimulation Induces the Release of Proinflammatory Cytokines by Melanoma Cells. It has been reported that CD40 ligation induces the secretion of cytokines such as IL-8 and IL-6 from human keratinocytes, fibroblasts, monocytes, B cells, and dendritic cells (reviewed in Refs. 1–3). Because MMs are capable of producing IL-8, IL-6, TNF-α, and GM-CSF among other T-cell chemotactic or T-cell-activating cytokines (15, 25), we analyzed the effects of CD40 ligation on cytokine secretion by the human MM cell line LB39-Mel (Fig. 2). LB39-Mel showed no constitutive expression of IL-8 or IL-6 and only low basal levels of GM-CSF and TNF-α (Fig. 2). Moreover, none of these cytokines could be detected in the supernatants of CD40-transfected or control L-cells cultured alone (Fig. 2). CD40L+L-cells stimulated LB39-Mel to secrete high levels of IL-8 and IL-6 (4–7 ng/ml) and GM-CSF (30 pg/ml) secretion was further enhanced (TNF-α, 20 ng/ml; GM-CSF 60 pg/ml) by IFN-γ priming (Fig. 2, C and D). Similar results were obtained with the human MM cell lines LB373, MM1, and MIRW stimulated with CD40L+L-cells and MIRW or LB39-Mel stimulated with sCD40L, except that GM-CSF and TNF-α were not detectable in MIRW (data not shown). Of note, IFN-γ alone had no effect on the basal production of IL-8, IL-6, TNF-α, or GM-CSF in any of the MM cell lines studied (Fig. 2 and data not shown). We conclude that CD40 ligation stimulates specifically the release of the proinflammatory cytokines IL-8, IL-6, and, in some cases, TNF-α and GM-CSF from CD40-positive human melanoma cells, whereas the production of other cytokines/growth factors such as basic fibroblast growth factor, transforming growth factor β1, IL-10, and IL-1β is not affected (data not shown).

CD40-mediated Signals Lead to Activation of the Transcription Factor NF-κB in Human Melanoma Cells. There is evidence that CD40 ligation results in the mobilization of NF-κB in B cells and nonhematopoietic cells (26, 27). Because transcription of the genes of

![Table 1: Cytokine-mediated regulation of CD40 expression on human melanoma cells](image_url)
CD40 Stimulation Enhances Tumor-specific CTL-mediated Killing of Melanoma Cells. Recently it has been demonstrated that CD40/CD40L-mediated signals are involved in the induction of CTLs (4–8). Moreover, CTLs recognize melanoma-specific antigens in a MHC class I-restricted fashion, and the expression levels of ICAM-1 and MHC class II molecules are crucial for adhesion, recognition, and lysis of target cells by CD8<sup>+</sup> CTLs (29). We, therefore, investigated whether the CD40 mediated up-regulation of these molecules (Fig. 4) affects melanoma cell lysis by CD8<sup>+</sup> CTLs. Cytotoxicity assays were performed using as targets the HLA-A2<sup>+</sup> MelanA/MART-1<sup>+</sup> human melanoma cell line LB39-Mel stimulated with CD40L or with CD40L<sup>-</sup> L-cells (data not shown). On the other hand, no CD40-mediated alterations in the surface expression of CD95, CD95L, B7-1, B7-2, CD44, CD40L, or CD40 could be detected (data not shown).

CD40 Ligation Up-Regulates Immunorelevant Surface Molecules on Melanoma Cells. APC triggering of CD40 by CD40L leads to the up-regulation of molecules involved in T-cell adhesion, antigen presentation, and activation, such as ICAM-1, MHC class I and II molecules, costimulatory molecules of the B7 family, and CD40 (reviewed in Refs. 1 and 2). Because MM cells are also capable of expressing some of these molecules, we investigated the effects of CD40 ligation on their expression of ICAM-1, MHC class I and II, B7-1, B7-2, CD95, CD95L, CD44, CD40L, and CD40 by FACS. MM cells were either untreated or primed with IFN-γ (200 units/ml, 24 h) and then incubated for 48 h with sCD40L, preincubated with the blocking mAb 39-1.106 or appropriate isotype control mAb (Fig. 4). MIRW constitutively expressed low level of surface MHC class I molecules (cMFI = 80; Fig. 4A). MIRW stimulated with sCD40L without IFN-γ priming showed only a weak up-regulation of MHC class I molecules (cMFI = 100). By contrast, after IFN-γ priming, CD40 stimulation induced a 2-fold up-regulation (cMFI = 240) of MHC class I molecules compared to MIRW primed with IFN-γ alone (cMFI = 115; Fig. 4A).

The expression level of MHC class II molecules on MIRW melanoma cells was affected analogously by CD40 stimulation (Fig. 4B). The cMFI of MHC class II was elevated 1.5-fold after the combined IFN-γ/CD40 stimulation (cMFI = 68), compared to IFN-γ priming alone (cMFI = 40; Fig. 4B). Of note, MIRW did not express MHC class II molecules in the absence of IFN-γ as detected by FACS (Fig. 4B). Likewise, ICAM-1 levels were elevated 3-fold after IFN-γ/CD40 stimulation (cMFI = 700), compared to basal levels (cMFI = 180; Fig. 4C). An enhanced shedding of soluble ICAM-1 paralleled this increase in surface ICAM-1 expression as determined by specific ELISA (Fig. 4D). All effects were specific for CD40 because they were reduced or inhibited by preincubation of sCD40L with the anti-CD40L mAb 39-1.106 but not with an isotype control mAb (Fig. 4). Comparable results were observed with the melanoma cell line LB39-Mel stimulated with sCD40L or with CD40L<sup>-</sup> L-cells (data not shown). On the other hand, no CD40-mediated alterations in the surface expression of CD95, CD95L, B7-1, B7-2, CD44, CD40L, or CD40 could be detected (data not shown).
fheshion was used as the effector population. Peptide specificity of CTL clone LB373-CTL-246/15 was confirmed using HLA-A2 transgenic T2 target cells pulsed with 1 μM MelanA/MART-1 peptide 27–35 (Fig. 5A, closed circles) or left untreated (Fig. 5A, open circles). Importantly, CD40 ligation following IFN-γ priming markedly enhanced the susceptibility of LB39-Mel melanoma cells to specific lysis by CTL clone LB373-CTL-246/15 (28% specific lysis; Fig. 5B, closed squares) as compared to unstimulated MM (12% specific lysis; Fig. 5B, open circles). Stimulation of LB39-Mel with IFN-γ alone (16% specific lysis; Fig. 5B, open squares) or CD40L L-cells alone (17% specific lysis; Fig. 5B, closed squares) had only minor effects on CTL lysis. Similar results were observed with LB39-Mel stimulated with sCD40L or CD40L L-cells (data not shown). This indicates that CD40 ligation on human melanoma cells in the presence of IFN-γ may play an important role in augmenting tumor lysis mediated by CD8+ CTL specific for melanoma antigens presented in the context of MHC class I molecules.

**CD40 Ligation Induces Growth Inhibition and Apoptosis in Human Melanoma Cells.** Controversial effects of CD40 triggering on the growth of nontransformed and of transformed cells have been described. Although CD40 stimulation of normal B cells leads to enhanced cell proliferation (reviewed in Refs. 1 and 2), growth inhibition and apoptosis are observed in B lymphoma and myeloma (11, 30, 31). To examine CD40-mediated effects on the growth and survival of human melanoma cells, we determined the proliferation and viability of MM cells and performed DNA fragmentation assays. LB39-Mel, either untreated or primed with IFN-γ for 24 h, were washed extensively, seeded at 3 × 10^5 cells in six-well plates and cultured either with medium alone, or stimulated with medium containing sCD40L (Fig. 6, A–D) or with CD40L L-cells (Fig. 6E). After 72 h, the cultures were inspected by inverted microscopy and photomicrographs were taken (Fig. 6, A–D). When compared to LB39-Mel cultured in medium (Fig. 6A), a treatment with sCD40L (Fig. 6B) or IFN-γ (Fig. 6C) alone had only moderate effects on cell density and cell morphology. By contrast, following IFN-γ priming, sCD40L treatment led to dramatic decrease in density and numbers of MM cells (Fig. 6D).

To confirm these results, MM cell proliferation was assessed by [3H]thymidine incorporation (Fig. 6E). Forty-eight-h coculture of LB39-Mel with mitomycin C-treated CD40L+ L-cells inhibited cell proliferation by 50%, as compared to MM cells cocultured with nontransformed L-cells or cultured in medium alone (Fig. 6E). IFN-γ priming further increased the CD40 induced inhibition of MM proliferation up to 70%, whereas IFN-γ alone had no effect (Fig. 6E). Addition of either sTNF-R1 (0.5 μg/ml), which effectively neutralizes the activity of TNF-α released by MM, or of APO1/Fas-immunoglobulin (20 μg/ml), a biologically active inhibitor of FasL activity, did not influence the CD40-mediated growth inhibition (data not shown). Similar observations as shown in Fig. 6, A–E, were made with MIRW (data not shown).

Inverted microscopy of CD40-stimulated MM also revealed morphological changes associated with apoptotic cell death-like membrane protrusion (Ref. 32; Fig. 6D). Indeed, FACS analysis of propidium iodide-stained MIRW cells, cocultured for 48 h with CD40L+ L-cells, demonstrated a 30% decrease in MM cell viability (data not shown), IFN-γ priming did not alter this effect. To verify whether CD40 ligation induces apoptosis in MM, we analyzed DNA preparations by agarose gel electrophoresis. Fragmentation of DNA was observed when MIRW were CD40 stimulated with sCD40L for 48 h, either with or without IFN-γ priming (data not shown). In contrast, pretreatment with IFN-γ alone or culture for 48 h in medium had no effect (data not shown). DNA fragmentation in MM cells was also assessed by enzymatic incorporation of fluorescein-labeled dUTP into DNA strand breaks and subsequent FACS analysis. FITC-dUTP-labeled DNA was detected when MIRW were cocultured for 72 h with sCD40L, either primed with IFN-γ or left untreated (Fig. 6F). Similar results were observed when LB39-Mel cells were stimulated with either CD40L+ L-cells or sCD40L (data not shown). The presence of sTNF-R1 (0.5 μg/ml) or APO1/Fas-immunoglobulin (20 μg/ml) during the entire period of CD40-stimulation did not prevent the induction of apoptosis (data not shown).

**DISCUSSION**

CD40 expression on human melanoma (MM) was described previously (17, 18), but little was known about its biological relevance. Here, we report the functional expression of CD40 on a subset of MM in situ as well as by some MM cell lines in vitro. Specifically, we demonstrate a gradual loss of CD40 expression during melanoma progression in situ. We also found human MM cell lines established from immunogenic MM but not from advanced-stage metastatic tissue positive for the CD40 antigen, thus paralleling the down-regulation of CD40 expression on MM metastases in situ. These results are in line with previous reports that 50% of MM cell lines generated from primary tumors, but only a minority of cell lines derived from metastases express CD40 (18). In CD40-negative MM, the expression of this antigen was increased after stimulation with IFN-γ or TNF-α but not with IL-1β or CD40L. By FACS analysis, we found none of the MMs positive for CD40L (CD154) using two different antibodies (39-1.106 and TRAP). We, therefore, cannot support the theory of an autocrine CD40-CD40L stimulation loop in MM, as suggested by van den Oord et al. (17).

Herein, we demonstrate that CD40 on human MM cells is functional because CD40 ligation stimulates their release of the proinflammatory cytokines IL-6, IL-8, GM-CSF, and TNF-α. We further demonstrate that cross-linking of CD40 on human MM up-regulates their surface expression of ICAM-1 and MHC class I and class II molecules, thus paralleling observations made in APCs (reviewed in Refs. 1 and 2). However, in contrast to APC, a CD40-mediated up-regulation of B7-1, B7-2, CD44, or CD40 (reviewed in Refs. 1 and 2) was not detected. Both the CD40-induced cytokine secretion and surface
molecule expression were enhanced by IFN-γ priming, which is most likely due to the IFN-γ-mediated up-regulation of CD40.

Our finding that some CD40-triggered MM cell lines release substantial amounts of IL-6, IL-8 (MIRW, LB39-Mel, MM1, and LB373), and GM-CSF and TNF-α (LB39-Mel, MM1, LB373) may have consequences for the generation of MM-specific immune responses. For example, murine MMs transfected with the GM-CSF or IL-6 gene form significantly smaller tumors and are more likely to be rejected than untransfected controls (33–35). The CD40-induced secretion of IL-8 could also augment the interactions of MM and T cells because a major biological activity of IL-8 is the attraction and activation of T cells to sites of specific immune responses (36). Additionally, we find that TNF-α secretion is enhanced in some CD40-stimulated MMs, which could exert direct antineoplastic effects on those tumor cells (37).

We wanted to characterize the mechanisms by which CD40 ligation up-regulates the above described surface molecules and cytokines in MM. Our finding that CD40 signals lead to NF-κB mobilization in human MM suggests that the CD40-induced events are mediated, at least in part, through activated NF-κB, as has been shown in other cell types (reviewed in Ref. 16). Because TNF-α activates NF-κB (16) and up-regulates the expression of ICAM-1, HLA class I and class II and distinct cytokines in MM (38), it is possible that these CD40-induced changes are due to TNF-α released from CD40-triggered MM. However, we consider this possibility unlikely because we also observed a CD40 stimulation of ICAM-1 and HLA class I and II, IL-6, and IL-8 in MIRW cells that did not secrete TNF-α following CD40 triggering. Furthermore, a sTNF-RI that effectively neutralizes TNF-α activity did not block the CD40-triggered response in MM.

The importance of CD40-CD40L interactions for the activation of specific CTL-effector populations has recently been described (4–7, 9). Here, we show that CD40 cross-linking leads to enhanced lysis of a HLA-A2+ and MelanA/MART-1-expressing melanoma cell line by a MelanA/MART-1-peptide specific CTL clone. It has been shown that the level of surface MHC class I expression on MM is crucial for their recognition by specific CTLs (39). Our observation that CD40 triggering up-regulates MHC class I molecules on MM may, thus, explain their enhanced susceptibility to specific CTL-mediated killing. This study also raises the possibility that in vivo CD40L-positive tumor-infiltrating CD4+ T cells may receive essential activation signals from CD40-positive MMs (4, 40, 41). Whether the secretion of soluble ICAM-1 molecules observed after CD40 stimulation in vitro has a negative impact on the susceptibility of MM to CTL lysis in vivo (42, 43) remains to be evaluated.

Furthermore, our study demonstrates that CD40 ligation inhibits the growth and subsequently leads to apoptosis of MM. This is in line with studies of CD40-mediated growth inhibition and apoptosis in transformed cells such as B lymphoma and myeloma (10, 11, 31). One explanation for CD40-mediated apoptotic signals is that the intracel...

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**Fig. 6.** CD40 ligation inhibits the proliferation of human melanoma cells via the induction of apoptosis. LB39-Mel cells were cultured at 37°C/8% CO₂ for 72 h when photomicrographs of representative cultures were taken by inverted microscopy (magnification, ×40). A, medium alone; B, medium plus sCD40L; C, 24 h prestimulation with 200 units/ml recombinant IFN; D, combined IFN-γ priming and sCD40L stimulation; E, LB39-Mel cells with or without IFN-γ (200 units/ml, 24 h) prestimulation were cocultured on monolayers of mitomycin C-treated CD40L+ L-cells or untransfected controls for 48 h at 37°C/8% CO₂. [³H]Thymidine (1 μCi/well) was added for the last 18 h of culture after which thymidine incorporation was measured by scintillation spectroscopy. Data are expressed as mean cpm ± SD of [³H]thymidine incorporation from quadruplicate cultures. Background proliferation of irradiated L-cells was <500 cpm. A representative experiment of four independent experiments performed with LB39-Mel or MIRW is shown. FACS analysis of terminal deoxynucleotidyltransferase-mediated dUTP-X nick end labeling staining of MIRW cells 48 h after the indicated treatments. Cells (2 × 10⁶) were fixed and permeabilized before direct labeling with FITC-dUTP. Comparable results were obtained with the melanoma cell line LB39-Mel stimulated with CD40L+ L-cells.
lular domain of CD40 shares structural homology with the death domains of p55TNFR and Fas (44, 45). Additionally, a CD40 signaling domain involved in growth inhibition has been identified recently (30). We consider it unlikely that growth inhibition and apoptosis are due to TNF-α induced by CD40-triggered MM because they are also observed in TNF-α-deficient MIRW or in the presence of neutralizing sTNF-R1. Moreover, CD40 stimulation did not alter or induce the expression of surface Fas or FasL on MM and a blocking APO-1/Fas-immunoglobulin had no effect on any CD40 triggered changes in MM. Taken together, these data indicate that CD40-induced growth inhibition and apoptosis in MM is not dependent on TNF-α or Fas/FasL expression.

Finally, our results support the notion that the loss of CD40 antigen during MM progression in vivo may represent yet another mechanism of tumor escape (43). Indeed, an inhibition of CD40/CD40L interaction by anti-CD40L mAb prevented tumor rejection of CD40

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Stimulation of CD40 on Immunogenic Human Malignant Melanomas Augments Their Cytotoxic T Lymphocyte-mediated Lysis and Induces Apoptosis

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