CD40 expressed on human melanoma cells mediates T cell co-stimulation and tumor cell growth

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Keywords: CD40, cell growth, co-stimulation, melanoma

Abstract
CD40 is a 50 kDa molecule, a member of the tumor necrosis factor/nerve growth factor receptor family. It is expressed on B cells, monocytes, dendritic cells and various malignant cells. While the critical relevance of this molecule in T cell-dependent B cell activation is already established, the biological role of CD40–CD154 interaction in non-hematopoietic cells is still unknown. Here we show that CD40 is functionally expressed on human melanoma-derived cell lines. No correlation between surface CD40 expression and the origin of the cell line, primary versus metastatic, was observed. Melanoma cells were shown to be able to co-stimulate TCR-triggered human T cells; moreover, because they do not express CD80 or CD86 co-stimulatory structures, the involvement of additional pathways have to be postulated. We have identified CD40 as one of the molecules involved in melanoma cell-mediated co-stimulation of anti-CD3-triggered human CD4+ T lymphocytes. In addition, a CD40-dependent pathway, able to enhance tumor cell proliferation at low serum concentrations, in vitro, has been shown to be functional in human melanoma cell lines.

Introduction
T cell activation requires both a specific signal delivered by antigenic peptide–MHC complexes to TCR molecules and a second signal, mediated by surface co-stimulatory molecules (1). TCR signaling in the absence of co-stimulation usually results in T cell inactivation or anergy, which is associated with a block in IL-2 gene transcription (2). Unlike TCR signaling, the delivery of co-stimulatory signals is neither antigen specific nor MHC restricted. One important co-stimulatory signal is transduced in T cells by the CD28–CTLA-4-dependent pathway (2). Although transfection experiments clearly demonstrate that the CD28 receptor–ligand system can be both necessary and sufficient for T cell co-stimulation, co-stimulatory pathways, virtually distinct from such ligand–receptor pairs, were described (3–7).

Several studies seem to indicate that tumor cells are able to deliver antigen-specific signals to T lymphocytes (8). In addition, a limited anti-tumor response has been demonstrated by the identification of tumor-reactive cytotoxic T lymphocytes (CTL) from peripheral blood of cancer patients (9). On the other hand, persistence and progressive growth of tumors indicate that the immune system is not generally competent to control the malignancy. In this context, a failure in co-stimulation has been suggested as a relevant escape mechanism for tumor cells from immune rejection (10).

CD40 is a 50 kDa membrane glycoprotein expressed on B cells (11), dendritic cells (12) and monocytes (13), involved in functional interactions between B and T lymphocytes (14). This molecule is a member of the tumor necrosis factor/nerve growth factor receptor family (15) which includes molecules like CD27 (16), CD30 (17) and CD95 (18) antigen, all of them critical for the regulation of both proliferation and apoptosis of hematopoietic cells (15). Murine and human forms of a receptor pair, were described (3–7).

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importance of the CD40 molecule-mediated interactions in B cell maturation. On the other hand, CD40-dependent pathways were observed to orchestrate the response of regulatory T cells both during their development (25,26) and their encounter with the antigen (6,14,27,28).

Functional expression of CD40 has been already described on a wide range of non-hematopoietic cell types, but the biological significance of CD40–CD154 interaction in normal as well as in transformed non-hematopoietic cells is still undefined. Thymic epithelial cells (29), keratinocytes (30), fibroblasts derived from the lung, gingiva, synovium and dermis (31), and vascular endothelial cells (32) were shown to express CD40 molecule. This antigen was also observed on a variety of tumors including nasopharyngeal (33), bladder, breast and colon carcinoma (34–36); moreover, human melanoma cells were also described to express the CD40 molecule (37,38).

Adaptive transfer of tumor-infiltrating lymphocytes has been demonstrated to mediate tumor regression in metastatic melanoma (39); in addition, spontaneous regressions occur more frequently in malignant melanoma than in other types of malignancies (40). In this context, the possible role of CD40-mediated pathways in the recruitment of functional immune effectors, capable of controlling tumor growth and progression, has been not yet analyzed.

In this study we investigated whether the CD40 molecule, functionally expressed on human melanoma cell lines, could act as a co-stimulatory structure in the presence of TCR triggering. The role of CD40-dependent pathways in tumor cell proliferation has been also analyzed.

Methods

Melanoma lines and T lymphocyte populations

Cultures of human melanoma cells were established as reported (41). In brief, tumor tissue, suspended in DMEM containing 20% FCS and supplemented with penicillin-streptomycin (100 IU/ml and 100 µg/ml), gentamycin (80 µg/ml) and L-glutamine (all purchased from Gibco, Paisley, UK), was minced with scalpels to a fine suspension which was repeatedly pipetted and centrifuged at 150 g for 5 min at room temperature. Cells were then re-suspended in fresh complete medium and cultured in six-well microtiter plates (Falcon, Seattle, WA) at 37°C in a humidified incubator with 5% CO2.

T lymphocytes were isolated from peripheral venous samples obtained from normal healthy donors, as previously described (42). Briefly, small, resting T lymphocytes were purified by using a Percoll density gradient after removal of B cells and monocytes by plastic and nylon wool adherence. The recovered high buoyant density population was always >97% CD3+, CD56-. The purity of T cell preparations was assessed by using immunofluorescence and phytohemagglutinin stimulation. To prepare CD4+ and CD8+ subpopulations, T cells, purified as described, were incubated with saturating concentrations of anti-CD4 or anti-CD8 mAb obtained from OKT4 and OKT8 hybridomas (both IgG2a), provided by ATCC (Rockville, MD). The labeled cells were removed by using magnetic beads coated with sheep anti-}

mouse Ig (Dynal, Oslo, Norway) and a samarium cobalt magnet. The depletion procedure was repeated twice. Less than 2% of the remaining cells expressed the antigen for which they were depleted, as assessed by immunofluorescence and FACS analysis.

mAb, immunofluorescence and flow cytometry

The anti-CD3 mAb CLB-CD3/4E (IgE) (43) and the anti-CD40 inducer mAb 14G7 (IgM) (44) were a gift of Dr R. van Lier (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands), whereas the control mAb VF20-VT43 (IgG2a) and KS5 (IgM) were a generous gift of Dr S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY). The anti-CD40 mAb 89 and 3C6 (both IgG1), and the anti-CD154 mAb TRAP-1 (IgG1) were purchased from Beckman-Coulter (Fullerton, CA) and PharMingen (San Diego, CA) respectively. FITC- and phycocerythrin-labeled mAb against CD3, CD4, CD8, CD14, CD19, CD56, HLA-DR and isotype-matched controls were purchased from Becton Dickinson (Mountain View, CA), and used to check purity of cell preparations by immunofluorescence. To define the phenotype of melanoma cell lines obtained in our laboratory, FITC-labeled anti-HLA class I W6/32 mAb (IgG2a), obtained from Sigma-Aldrich Italia (Milan, Italy), anti-high-molecular-weight melanoma-associated-antigen (HMW-MAA) 763.74 mAb (IgG1) and anti-low-molecular-weight melanoma-associated-antigen (LMW-MAA) HMB45 mAb (IgG1), both a generous gift of Dr S. Ferrone, were used. In addition, FITC-labeled anti-CD54, 6.5B5 mAb (IgG1) purchased from Dako Italia (Milan, Italy) and anti-CD44, G44-26 mAb (IgG2b) obtained from PharMingen were employed. Flow cytometry and data analysis were performed by using a Becton Dickinson FACS Vantage flow cytometer and CellQuest analysis software, as described (45).

Proliferation assay

Melanoma cells (5×10⁴) were cultured in 24-well microtiter plates (Falcon, Seattle, WA) in the presence of DMEM containing different percentages of FCS, as indicated in Results. Human rIFN-γ was purchased from Sigma-Aldrich Italia. Resting peripheral T cells (2×10⁵) were activated with anti-CD3 mAb CLB-CD3/4E, in 96-well flat-bottomed microtiter plates (Falcon, Seattle, WA) in the presence of different percentages of 100 Gy irradiated melanoma cells, as indicated in Results.

Cultures were incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO2 and pulsed with 0.5 µCi/well ³H-thymidine for the final 16 h. The incorporation of labeled nucleotide was determined by scintillation counting, after harvesting, using an automatic cell harvester. All tests were performed in the presence of RPMI 1640 medium supplemented with 5% heat-inactivated FCS.

Statistical analysis of data was performed using Student’s t-test.

Results

Human melanoma cells express a functional CD40 molecule

CD40 expression has been described on human melanoma cells (36,37). To examine whether CD40–CD154 binding might
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CD40 antigen expressed on the surface of human melanoma cell lines is able to co-stimulate TCR-dependent T cell proliferation

To examine whether melanoma cells could co-stimulate TCR-triggered T lymphocytes, we cultured human T cells, obtained as described in Methods, with anti-CD3 mAb in the presence of different percentages of irradiated melanoma cells. To overcome the need for cross-linking of anti-CD3 by Fc receptors (usually not expressed on the surface of these cells) we used anti-CD3 mAb CLB-CD3/4E (IgE) as 1/4000 diluted ascites. This mAb can stimulate purified peripheral blood T cells in the absence of cross-linking (43). The involvement of a CD40-mediated pathway in the co-stimulation of TCR-dependent T cell proliferation has already been described by us and others (6,25). To investigate whether CD40 might play a role in melanoma-mediated co-stimulation of CD3-dependent T cell proliferation, the cultures were performed in the presence of saturating concentrations of anti-CD40 mAb 89 or of the control mAb VF20-VT43.

As shown in Fig. 3, CD40+ melanoma cell lines were able to co-stimulate TCR-triggered human T cells. Figure 3(A) clearly shows that <5% of irradiated CD40+ melanoma cells was enough to obtain an optimal co-stimulation. In addition, Fig. 3(B) shows that the presence of anti-CD40 mAb 89, but not of the control mAb, significantly affected the ability of the CD40+ melanoma cells to co-stimulate TCR-triggered resting T cells (P < 0.004). An inhibition rate ranging from 35 to 65%, as evaluated in five independent experiments, was observed. Intriguingly, CD40+ melanoma cell lines were also able to co-stimulate TCR-triggered T cells, even though at lower efficiency (P < 0.02), as compared with the CD40+ counterpart (Fig. 3C).

CD40 was described to preferentially co-stimulate TCR-triggered CD4+ human T cells (6,25). Therefore, we performed experiments in which purified CD4+ or CD8+ T cells were tested for their ability to be co-stimulated by CD40-expressing melanoma cells. As shown in Fig. 4, CD40+ melanoma cells were able to co-stimulate both CD4+ and CD8+ human T cells, whereas anti-CD40 mAb was observed to significantly affect only CD4+ T cell proliferation (P < 0.006). Interestingly,
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Fig. 1. IFN-γ treatment is able to up-regulate, but not to induce, CD40 expression on human melanoma cell lines. The CD40⁺ melanoma line PES43 (A and B) and the CD40⁻ line CIMA62 (C and D) recovered after 48 h of incubation in the presence of medium or rIFN-γ (250 IU/ml), as indicated, were stained with saturating concentrations of FITC-labeled anti-CD40 mAb 3C6 (A and C) or anti-class I mAb W6/32 (B and D). Control staining with FITC-conjugated isotype IgG is shown.

CD40⁻ melanoma cell lines maintained their ability to co-stimulate, even if at a lower extent, both CD4⁺ as well as CD8⁺ T lymphocytes (not shown).

These results indicate that CD40 antigen expressed by melanoma cells might play a role in the co-stimulation of TCR-triggered CD4⁺, but not of CD8⁺ human T cells; this observation, the inability to show evidence of CD80 or CD86 antigens on melanoma-derived cell lines and the maintained co-stimulatory ability of CD40⁻ melanoma cells, suggest the involvement of additional co-stimulatory pathways.

CD40 triggering is able to significantly increase melanoma cell proliferation at low serum concentrations

Our data demonstrate that CD40-dependent intracellular pathways are functional in melanoma cells, being able to significantly modify the surface phenotype of tumor cells (Fig. 2). Therefore, we analyzed whether CD40-dependent signals could also affect the growth rate of cultured melanoma cells. The anti-CD40 14G7 mAb was used as a CD40-specific triggering element. Proliferation levels were measured by using a classic thymidine incorporation assay after a 48 h incubation period performed in the presence of different serum concentrations. Melanoma cells, cultured in the presence of decreasing percentages of serum, also revealed decreasing proliferation levels. In this context no differences were observed in the proliferation levels of the CD40⁺ melanoma-derived cell lines as compared to the CD40⁻ counterpart (not shown). Interestingly, as shown in Fig. 5, in each experimental condition the presence of the anti-CD40 inducer mAb 14G7 significantly enhanced (P < 0.01) the proliferation of CD40⁺ melanoma cells. No interference was observed in the presence of the control mAb KSS. The effect appeared to be more relevant in the presence of low serum concentrations. Also in the absence of serum, CD40 triggering was able to induce a significant increase in melanoma cell proliferation levels (Fig. 5C). As shown in Fig. 5(D), anti-CD40 treatment was unable to affect the proliferation levels of CD40⁻ melanoma cell lines at all serum concentrations.

Our results show that a CD40-mediated pathway could be involved in melanoma cell proliferation, especially when 'non-optimal' culture conditions were employed. The CD40 molecule therefore might act both as a co-stimulatory structure involved in TCR-dependent T cell proliferation and as a signaling element which plays a critical role in the regulation of melanoma cell growth. Serum lacking and probably stressing conditions seem to potentiate such effect in vitro.

Discussion

CD40-CD154-mediated interactions not only are important in the regulation of the adaptive immune response, but are also involved in the inflammatory response, the regulation of
adhesion molecules on epithelial cells, and the induction of inflammatory cytokines, chemokines and IL-12 (46,49,50).

We have shown here that the CD40 molecule, functionally expressed on human melanoma-derived cell lines, could play a role in the complex interactions underlying recruitment and/or expansion of ‘tumor recognizing’ immune effectors, as well as in the biochemical pathways involved in the regulation of tumor cell growth in vitro.

Indeed, melanoma cells, similarly to keratinocytes or endothelial cells (51), are able to act as ‘non-professional antigen-presenting cells’. As cultured melanoma cells do not express...
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Fig. 4. CD40 expressed on human melanoma cell lines preferentially co-stimulate CD3 triggered CD4+ but not CD8+ T lymphocytes. T lymphocytes or CD4+, CD8+ subsets, obtained as described in Methods, were incubated with irradiated melanoma cell line ALVE39 in the presence of medium (\(\text{H}\)), anti-CD3 mAb CLB-CD3/4E (\(\text{E}\)) (1:4000 ascites dilution) alone or plus anti-CD40 mAb 89 (\(\text{F}\)) (2 \(\mu\)g/ml) or the control mAb VF20-VT43 (\(\text{G}\)) (2 \(\mu\)g/ml). (A–C) Results obtained by using total T lymphocytes, CD4+ or CD8+ subsets respectively. \[^{[3]}H\]Thymidine incorporation was evaluated after a 3 day incubation period without background subtraction. Results are presented as mean c.p.m. of triplicate cultures; SD was always <15%. No proliferation was observed after incubation of T cells with or without anti-CD3 in the absence of irradiated melanoma cells. Data are representative of four independent experiments.

Fig. 5. CD40 triggering can increase proliferation levels of human melanoma cell lines. (A–C) Proliferation data obtained by culturing CD40+ melanoma cell line PES43 in the presence of 10% FCS, 2% FCS or without serum respectively. Culture conditions were described in Methods. Data were confirmed by using three CD40-expressing melanoma cell lines in, at least, three independent experiments. (D) Data obtained by using as control the CD40− melanoma cell line CIMA62 at 2% FCS concentration. No differences were observed by using 10% FCS or without serum.
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CD40 or CD86, the co-stimulatory signals should be provided by other molecules. This study identifies CD40 as one of the molecules involved in melanoma cell-mediated co-stimulation of anti-CD3-induced human T cell proliferation. In addition, the preferential role of this molecule in delivering accessory signals to CD4+ T lymphocytes (6,26) was confirmed.

Recent data (52) show a lack of immune response to autologous tumors in CD154-deficient mice, strongly reinforcing the central role of the CD40–CD154 pathway in T cell priming. It is not clear if CD40–CD154 interaction could also affect the priming of CD8+ T cells, but the observation that the antiviral CD8-dependent CTL memory responses appear defective in CD154-deficient mice seems to suggest a requirement for CD154-mediated signals in the establishment of CTL memory.

CD40-dependent pathways have been reported to be essential in delivering helper signals critical for CTL response (53). In addition CD40 triggering is able to potentiate cytotoxic activity of human CTL clones against melanoma antigen MART-1 (38); therefore, the possible involvement of this pathway in the recruitment and/or expansion of tumor recognizing CD8+ cells could also be suggested.

CTL response requires MHC class I-restricted antigen presentation (54). The presence of class I-defective tumor clones was already described in cancer progression and indicated as critical for the lack of an efficient CD8-dependent cytotoxic activity (54,55). Our recent results (56) proposed the CD40–CD154 interaction as relevant for NK triggering in the absence of MHC class I surface expression. Moreover, the observation that CD40 can be revealed on HLA class I+, as well as negative melanoma cell clones (our unpublished results), suggests a potential role of this molecule in the recruitment of functional NK effectors able to eliminate class I+/CD40+ tumor clones.

In melanoma model it seems that in vitro the CD40 molecule can behave as a co-stimulatory element as well as a signaling structure able to enhance tumor cell proliferation. Therefore a new and more complex scenario for tumor-mediated T cell co-stimulation in vivo can be hypothesized.

In B cells both cell growth and apoptosis appear to be induced by CD40-mediated pathways, depending from the different activation state of B lymphocytes (49). Less defined and still controversial appears to be the available data on the possible role of CD40 in the regulation of apoptosis in non-hematopoietic cells (35,36,38,57), while a CD40-dependent pathway has been described in the differentiation of human keratinocytes in vitro (58).

The above observations point out the problem of the possible involvement of CD40 molecules in the biological mechanisms underlying human melanoma progression. In this context, the ongoing long-term follow-up studies of CD40 expression in primary and metastatic melanoma tissues are expected to effectively address the possible relevance of CD40 surface levels as a prognostic marker for human melanoma.

In most metastatic melanoma lesions, in spite of the presence of anti-melanoma-specific CTL clones in vivo, poor evidence of tumor regression was observed (59). These data suggest that tumor escape mechanisms hinder T cell-mediated immune response in vivo. On the other hand, the critical role of CD40-dependent pathways in the regulation of both the innate (50,56) as well as adaptive (46,50) immune response suggests that we should investigate the potential ability of CD40 antigen to provide a useful frame for the establishment of an effective anti-tumor immune response. Therefore, the analysis of the biological mechanisms underlying the complex balance between CD40-dependent immune co-stimulation and tumor survival could provide novel tools for innovative immune therapeutic approaches.

Acknowledgments

Dedicated to the memory of Ciro Manzo. We are in debt with Dr. S. Ferrone for the generous gift of mAb, continuous helpful discussion and critical reading of the manuscript. We also thank Drs. S. Zappacosta, G. Matarrese, G. Terrazzano and E. de Angelis for critical reading of the manuscript. Supported by Italian Ministero della Sanità grant no. ICS150.1/R/F97.107.

Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>HMW-MAA</td>
<td>anti-high-molecular-weight melanoma-associated-antigen</td>
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<tr>
<td>LMW-MAA</td>
<td>anti-low-molecular-weight melanoma-associated-antigen</td>
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