Expression of Functional CD40 on Human Osteosarcoma and Ewing’s Sarcoma Cells

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ABSTRACT

CD40, a membrane glycoprotein of the tumor necrosis factor receptor family, is expressed by several tumor types, including B-cell lymphomas, carcinomas, and melanoma, but little is known concerning its expression by sarcoma. We used flow cytometry to analyze the expression of CD40 in human cell lines derived from 12 osteosarcomas, 6 Ewing’s sarcomas, and 5 rhabdomyosarcomas. Detectable CD40 levels ranged from low to very high were found in one-third of osteosarcomas, whereas five of six Ewing’s sarcomas expressed intermediate levels of CD40; all rhabdomyosarcomas were CD40-negative. At the tissue level, two of eight primary high-grade osteosarcomas showed CD40-positive immunostaining. Osteosarcoma cells and Ewing’s sarcoma cells expressing CD40 were treated with recombinant soluble CD40 ligand to analyze CD40 function. Treatment with soluble CD40 ligand increased the level of apoptotic cells and stimulated the transcription of matrix metalloproteinase 9 gene, enhancing matrix metalloproteinase 9 enzyme secretion. The results indicate that in human osteosarcoma and Ewing’s sarcoma, CD40 is a functional receptor whose engagement can have opposite effects on tumor cell survival and malignancy.

INTRODUCTION

CD40 is a type I membrane glycoprotein of the TNF receptor family (1). CD40 is involved in the genesis of the immune response. It is highly expressed by B lymphocytes, and its engagement by a ligand (CD40L or CD154) expressed by T lymphocytes plays an essential role in proliferation, survival, and immunoglobulin production (2). Other elements of hematopoietic origin that express CD40 include macrophages and dendritic cells (1, 3); however, CD40 is not restricted to cells of the immune system. Epithelial cells in several districts and organs express CD40; a partial list of such cells includes keratinocytes (4, 5), the basal layer of the nasopharynx, gastrointestinal and bronchial mucosa, and several exocrine glands, such as the salivary glands, sweat glands, mammary glands, and pancreas (1). Consequently, CD40 expression has been found in carcinomas of different origin, including those of the urinary bladder, colon, prostate, breast, and lung (1). Normal mesenchymal cells are also known to express CD40; for example, CD40 expression was demonstrated on synoviocytes, fibroblasts, and endothelial cells both in vitro and in vivo, particularly during tissue regeneration and inflammatory responses (6–9). With the exception of Kaposi’s sarcoma and tumors of vascular origin (10), little is presently known concerning functional CD40 expression in human sarcomas. For this reason, we studied a series of cells derived from human musculoskeletal sarcomas, including osteosarcomas, Ewing’s sarcomas, and rhabdomyosarcomas.

MATERIALS AND METHODS

Cell Culture. A panel of cell lines from human musculoskeletal sarcomas of different histogeneses was used. RD/18 and CCA were derived from embryonal rhabdomyosarcomas; RMZ-RC2, SJRH4, and SJRH30 were derived from alveolar rhabdomyosarcomas (11). SJRH4 and SJRH30 were kind gifts from Dr. David N. Shapiro (St. Jude Children’s Hospital, Memphis, TN) and Angelo Rosolen (Department of Pediatrics, University of Padua, Padua, Italy). Osteosarcoma cell lines Saos-2, U-2 OS, and MG-63 were obtained from the American Type Culture Collection (Rockville, MD), whereas the other osteosarcoma cell lines were established at the Rizzoli Institute (Bologna, Italy) and have been characterized previously (12). Ewing’s sarcoma cell lines SK-ES and RD-ES and the Askin’s tumor cell line SK-N-MC were obtained from the American Type Culture Collection. TC-71 and 6647 were gifts from Timothy J. Triche (Children’s Hospital, Los Angeles, CA). LAP-35 was established at the Rizzoli Institute from a patient with a peripheral neuroectodermal tumor (13). Rhabdomyosarcoma cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum. Osteosarcomas and Ewing’s sarcomas were cultured in Iscove’s modified DMEM and 10% fetal bovine serum.

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3 The abbreviations used are: TNF, tumor necrosis factor; CD40L, CD40 ligand; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-PCR; sCD40L, soluble CD40L; LMP1, latent membrane protein 1; NFκB, nuclear factor κB; TIMP, tissue inhibitor of metalloproteinase.
Flow Cytometry. Cells were harvested with 5 mM EGTA in calcium- and magnesium-free PBS and stained using a standard indirect immunofluorescence procedure with anti-CD40 monoclonal antibody M2 (14), a kind gift from Dr. Elaine K. Thomas (Immunex Corp., Seattle, WA), followed by a F(ab')2 FITC-conjugated antimouse immunoglobulin antibody (Kierkegaard & Perry, Gaithersburg, MD). After final washings, the cells were resuspended in PBS containing 1 μg/ml ethidium bromide to gate out the dead cells. A FACScan flow cytometer was used (Becton Dickinson, Mountain View, CA).

Immunohistochemistry. The expression of CD40 was analyzed at the tissue level in eight primary osteosarcomas, which were obtained from the Department of Oncology of the Istituti Ortopedici Rizzoli (Bologna, Italy). Tissue specimens were embedded in cryopreservative solution (OCT compound; Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C. Before immunostaining, H&E-stained tissue sections of each block were examined microscopically to confirm the presence and integrity of the tumor cells.

Immunostaining was performed using the avidin–biotin peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Tissue sections were fixed with paraformaldehyde and 4% PBS for 10 min at room temperature. After blocking the endogenous peroxidase activity by treatment with methanol and 1% hydrogen peroxide for 30 min, the sections were incubated with normal horse serum (Vector Laboratories) for 30 min at room temperature, followed by an overnight incubation at 4°C with the anti-CD40 monoclonal antibody M2 at a 1:200 dilution. After washing, the sections were subsequently incubated for 30 min at room temperature with a biotinylated horse antimouse antibody and avidin–biotin peroxidase complex (Vector Laboratories). The final reaction product was revealed by incubation with diaminobenzidine (Sigma, St. Louis, MO), and the nuclei were counterstained with Gill’s hematoxylin.

Sections of cell pellets from human osteosarcoma cell lines U-2 OS and OS-9 were used as positive and negative controls, respectively. In addition, for each sample, a negative control for immunostaining was carried out by replacing the primary antibody with normal horse serum.

Induction of Apoptosis by CD40L. Recombinant soluble trimeric human CD40L/leucine zipper fusion protein (sCD40L) produced in Chinese hamster ovary cells was a kind gift from Dr. Elaine K. Thomas. TNF-α was a kind gift from Dr. Günther R. Adolf (Ernst-Boehringer Institute, Vienna, Austria). CHX was purchased from Sigma (Milan, Italy). Preliminary titrations of sCD40L showed maximal effects at 1–2 μg/ml; no increase was obtained with 5–10-fold higher doses, whereas a significant decrease was observed at 0.1 μg/ml. sCD40L, TNF-α, and CHX were added to cells in 24-well plates (Falcon) for 24 h. Cells were then harvested from each well and stained with Hoechst 33342 (Merck, Milan, Italy), and apoptotic bodies were evaluated at high-power magnification in a Leica DM microscope. At least 400 elements were evaluated for each sample.

PCR. Total cellular RNA was isolated from untreated cells and from cells treated with 2 μg/ml sCD40L for 24 h using the guanidine isothiocyanate method. RNA concentration and quality were assessed by A260/280 nm and electrophoretic pattern, and then 1 μg of RNA was reverse-transcribed in a final volume of 30 μl by using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in the presence of oligodeoxynucleotidyl acid and deoxyribonucleotide triphosphate. cDNA (1 μl) was used for RT-PCR in a final volume of 25 μl using primer pairs for MMP-9 or MMP-2 (15) or for GAPDH (Clontech, Palo Alto, CA). Due to the very different amounts of transcripts for the three genes studied, for each primer pair, PCR products of CD40L-treated cells were compared with those of untreated cells in the exponential amplification phase (GAPDH, 20 cycles; MMP-2, 27 cycles; and MMP-9, 40 cycles). RNA was extracted from at least three independent experiments. cDNAs for each experiment were simultaneously tested in RT-PCR at least twice. Densitometric analysis of the RT-PCR-amplified products was performed on ethidium bromide-stained bands.

Detection of MMP-9 Activity. Modulation of MMP-9 secretion by sCD40L was evaluated on confluent monolayers of U-2 OS or RD/18 cells by replacing the culture medium with fresh medium with or without 2 μg/ml sCD40L. Supernatants were collected 24 h later. MMP-9 activity in supernatants was determined by the ELISA test Biotrak MMP-9 activity assay system (Amersham Pharmacia Biotech, Milan, Italy). The assay range was 0.5–64 ng/ml. The test was performed according to the manufacturer’s instructions. Briefly, standards and samples were incubated at 4°C overnight in microtiter wells precoated with anti-MMP-9 antibody. After extensive washings, any bound MMP-9 was activated by adding aminophenylmercuric acetate. Active MMP-9 was then detected through the activation of a modified urokinase proenzyme and the subsequent cleavage of its chromogenic peptide substrate. After a 6-h incubation at 37°C, the resultant color was read at 405 nm, and the concentration of active MMP-9 was determined by interpolation from the standard curve.

RESULTS

Expression of CD40 by Human Sarcoma Cells. Using a standard anti-CD40 monoclonal antibody and fluorescence-activated cell-sorting analysis, we found that CD40 was detectable on the surface of 5 of 6 Ewing’s sarcoma cell lines and 4 of 12 osteosarcomas, but not on rhabdomyosarcoma cells (Fig. 1). Osteosarcomas showed the broadest range of CD40 levels. The U-2 OS cell line expressed CD40 at the same order of magnitude as the B lymphoma cell line Raji, one of the highest CD40 expressers known. The OS-7 cell line was positive for CD40 expression, but at an intermediate level. The Saos-2 cell line showed bimodal CD40 expression, with half of the population reaching an intermediate level. The OS-10 cell line had borderline CD40 expression, with a small proportion of cells expressing very low levels of CD40. Ewing’s sarcoma cell lines seemed to be more homogeneous, showing intermediate levels of expression.

Expression of CD40 in Human Osteosarcoma Biopsies. The expression of CD40 in cell lines derived from Ewing’s sarcomas and rhabdomyosarcomas is in agreement with an immunohistochemical study (16) that reported that two-thirds of Ewing’s sarcomas express CD40, whereas rhabdomyosarcomas are CD40-negative.
**Fig. 1** Flow cytometric analysis of CD40 expression in human musculoskeletal sarcoma cells. *Shaded profiles*, cells incubated with anti-CD40 antibody. *Open profiles*, cells incubated with secondary antibody alone. CD40 expression of the human B lymphoma cell line Raji is reported for comparison. Data are representative of two to six independent experiments. In each panel, the *ordinate* represents the number of cells.
No data concerning CD40 expression in osteosarcomas were available; therefore, we examined a series of eight primary osteosarcomas obtained from the Department of Oncology of the Istituti Ortopedici Rizzoli. At the tissue level, CD40-positive immunostaining was found in two of eight (25%) primary high-grade osteosarcomas. When present, immunoreactivity was observed both in the cytoplasm and at the membrane level of tumor cells, whereas the extracellular matrix was invariably CD40-negative (Fig. 2, A and B). Human osteosarcoma cell line U-2 OS, which was used as a positive control for CD40 immunostaining, showed a positive reaction in the vast majority of cells (Fig. 2C), whereas no evidence of immunoreactivity was ever found in OS-9 human osteosarcoma cells (Fig. 2D).

Response of Sarcoma Cells to CD40L. Triggering the functional CD40 receptor has multiple effects on normal and transformed cells of different lineages (1). We decided to further investigate in sarcoma cells two biological responses that could be relevant from an oncological point of view, namely: (a) the induction of apoptosis (7); and (b) the acti-

Fig. 2 Immunohistochemical study of CD40 expression at the tissue level in human primary osteosarcomas. A and B respectively show a positive and a negative immunostaining for CD40 in two different high-grade osteosarcomas. C and D are respectively the U-2 OS cell line and the OS-9 cell line used as positive and negative control.
vation of MMPs (17). We used sCD40L to stimulate cell lines expressing CD40.

**Induction of Apoptosis by CD40L.** Apoptotic U-2 OS cells showing typical nuclear condensation or fragmentation were detected after treatment with sCD40L alone or in combination with TNF-α or CHX (Fig. 3, A–C). A quantitative analysis of apoptosis after the exposure of U-2 OS cells to sCD40L revealed a small increase in the proportion of apoptotic cells over that of untreated control cultures (Fig. 3D). A more significant increase was observed when sCD40L was added to cultures treated with TNF-α or CHX (Fig. 3D).

**Induction of MMPs by CD40L.** Induction of MMPs by CD40L has been recently demonstrated in human monocytes and in the human monocytic cell line THP-1 (17). We found that sCD40L stimulated MMP-9 gene transcription in three CD40-positive cell lines (OS-15, U-2 OS, and SK-ES) but not in CD40-negative cell lines (OS-15 and RD/18; Fig. 4A). The induction of MMP-9 mRNA by CD40L was confirmed in three independent experiments. The intensity of the ethidium bromide-stained bands was evaluated by densitometric analysis (Fig. 4B), showing a significantly higher intensity in CD40-positive cell lines treated with sCD40L as compared with that in untreated cells. Densitometric analysis of the housekeeping gene GAPDH demonstrated that comparable amounts of cDNA had been obtained for each cell line, regardless of sCD40L treatment (data not shown). In the CD40-positive U-2 OS cell line, no difference between sCD40L-treated and untreated cells was observed in the mRNA expression of a second MMP gene, MMP-2 (data not shown), indicating that sCD40L treatment selectively affected MMP-9 gene transcription.

To assess whether the increase in MMP-9 mRNA was followed by enzyme secretion, we analyzed cell supernatants with an ELISA system measuring active MMP-9 (see “Materials and Methods”). Fig. 5 shows that U-2 OS cells had a low basal production of MMP-9 that was significantly increased after treatment with sCD40L. No MMP-9 activity was detectable in supernatants conditioned by CD40-negative RD/18 cells either before or after sCD40L treatment (Fig. 5).

**DISCUSSION**

The analysis of CD40 expression in human musculoskeletal sarcoma cells revealed that one-third of osteosarcoma cell lines and five of six Ewing’s sarcoma cell lines expressed membrane CD40. A wide variation in CD40 levels was found among osteosarcomas; one osteosarcoma, U-2 OS, displayed a very high CD40 level, similar to that of B-cell lymphomas. The five CD40-positive Ewing’s sarcoma cell lines were more homogeneous at intermediate CD40 levels.

To the best of our knowledge, this is the first demonstration that human osteosarcomas can express CD40, both in *in vitro* cultured cells and at the tissue level (two of eight primary osteosarcomas were CD40-positive). Regarding Ewing’s sarcoma and rhabdomyosarcoma, our results with the cell lines are in good agreement with an immunohistochemical study (16) showing CD40 expression in a small series of Ewing’s sarcomas and peripheral neuroectodermal tumors (six of nine positive specimens) and no CD40 expression in nine rhabdomyosarcoma patients.

![Fig. 3 Induction of apoptosis by sCD40L in CD40-positive human osteosarcoma U-2 OS cells. Morphological features of apoptotic cells in cells treated with CD40L (A), TNF + CD40L (B), and CHX + CD40L (C) are shown. Arrowheads, fragmented nuclei; arrows, condensed nuclei. D, each bar represents the proportion of apoptotic cells evaluated in independent experiments by Hoechst 33342 staining and by microscopic counting of at least 400 cells after treatment for 24 h with (Ⅰ) or without (Ⅱ) sCD40L (1 µg/ml). Simultaneous treatments with TNF-α (1000 IU/ml) or CHX (50 µg/ml) were also evaluated. The results obtained with and without sCD40L were compared by the paired *t* test (*, *P* < 0.05; **, *P* < 0.01).](https://clincancerres.aacrjournals.org/content/6/8/1847/F3.large.jpg)
We showed that the functional consequences of CD40 engagement include stimulation of apoptosis and an increase in the transcription of an inducible metalloproteinase gene, MMP-9, a phenomenon previously observed only in monocytes (17). MMP-9 up-regulation by sCD40L was confirmed by detection of the active enzyme in culture supernatants. These data clearly indicate that CD40 expressed by sarcomas is a functional receptor that can modulate cell behavior. The analysis of CD40 function in cells of hemopoietic origin revealed a wide range of distinct responses (1); therefore, additional studies are now required to fully appraise the range of CD40 functions in human sarcomas.

Up-regulation of MMP-9 expression by EBV LMP1 has recently been described in human lymphoma cells (18). Transcriptional regulation of the MMP-9 gene was stimulated by LMP1 via NFκB, SP-1, and activator protein 1 binding sites. Engagement of CD40 is also known to induce activation of NFκB and of the Jun and Fos family transcription factors (19); therefore, we hypothesize that those factors might mediate the CD40-induced increase in MMP-9 observed in sarcoma cells. LMP1 and CD40 were not able to increase MMP-2 mRNA expression, probably because the MMP-2 promoter does not have a NFκB binding site (18).

A proapoptotic role of NFκB/Rel has been reported in some cell types (20). In addition, the balance between MMPs and their inhibitors (TIMPs) may also play a role in apoptosis. TIMP-2 overexpression has been shown to increase the resistance of melanoma cells to apoptosis (21), and TIMP-1 inhibited apoptosis in Burkitt’s lymphoma (22) and mammary cells (23). Thus, it is conceivable that stimuli that tip the MMP/TIMP balance in favor of MMP could also stimulate cell death by apoptosis.

An important issue at this point is to establish whether CD40 is merely a useful marker for certain types of tumors, or whether it bears a causal relationship to tumor onset and progression. The study of X-linked hyper-IgM syndrome patients carrying mutations in the CD40L gene recently uncovered an association with tumors of the pancreas, liver, and biliary ducts (24). It was hypothesized that the lack of CD40-CD40L interactions might prevent an effective antitumor immune response, thus facilitating early tumor progression. A critical role of CD40 in the genesis of immunity against solid tumors was also demonstrated by a study of mice genetically deficient in CD40 expression: cellular vaccines that confer immunity to a subsequent challenge with tumor cells to normal mice failed to protect CD40-deficient mice (25).

Different indications come from the study of established human solid tumors. First of all, several types of malignant tumor do express CD40 (10, 16, 26), and in the case of metastatic melanoma, a worse prognosis has been demonstrated for...
lesions containing CD40-positive cells in comparison with CD40-negative tumors (27). Our results indicate that CD40 engagement activates the transcription of a gene involved in tumor invasion and metastasis in tumor cells (28).

Taken together, these results suggest that CD40 might have opposing functions at different stages of tumor progression: apoptosis at the outset; and tumor invasion at later stages.

Unrelated reports in disparate tumor systems indicate that CD40 and its ligand might influence diverse biological activities of tumor cells, including cell adhesion, motility, differentiation, and death. We believe that additional investigations are required to fully appraise the role of CD40/CD40L in tumor progression and malignancy.

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