Cripto: A Novel Target for Antibody-Based Cancer Immunotherapy

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ABSTRACT

Cripto, a member of the epidermal growth factor-Cripto-FRL-Criptic (EGF-CFC) family, has been described recently as a potential target for immunotherapy (Adkins et al., J Clin Invest 2003;112:575–87). We have produced rat monoclonal antibodies (mAbs) to a Cripto 17-mer peptide, corresponding to the “EGF-like” motif of Cripto. The mAbs react with most cancers of the breast, colon, lung, stomach, and pancreas but do not react or react weakly with normal tissues. The mAbs inhibit cancer cell growth in vitro, and this effect was greater with cytotoxic drugs such as 5-fluorouracil, epirubicin, and cisplatin. The anti-Cripto mAbs prevent tumor development in vivo and inhibit the growth of established tumors of L5174T colon xenografts in Scid mice. The growth inhibitory effects with these mAbs may be greater than those described elsewhere, possibly because of IgM giving more effective cross-linking or binding to a different epitope (EGF-like region versus CFC region). The mechanism of inhibitory effects of the Cripto mAbs includes both cancer cell apoptosis, activation of c-Jun-NH2-terminal kinase and p38 kinase signaling pathways and blocking of Akt phosphorylation. Thus, Cripto is a unique target, and mAbs to Cripto could be of therapeutic value for human cancers.

INTRODUCTION

Human Cripto is a M, 36,000 molecule, classified in the epidermal growth factor-Cripto-FRL-Criptic (EGF-CFC) family, caused by the conservation of six cysteines in the central region (amino acids 77–113); however, there is little resemblance to EGF, and Cripto does not bind to any EGF receptors (1, 2). The EGF-CFC family includes human Cripto, mouse Cripto-1, cryptic Xenopus FRL-1, zebrafish one-eyed pinhead, and chick Cripto (3, 4). Cripto contains a signal sequence, a characteristic EGF-like domain, a second cysteine-rich region motif (CFC domain), and a hydrophobic COOH-terminus. Additionally, Cripto is a membrane-bound protein, anchored in the lipid bilayer of cell surfaces by a glycosyl-phosphatidylinositol (5), and acts as both cell surface coreceptors (6) and has activity when interrupted Cripto function and may be a useful approach to cancer immunotherapy (Min et al., J Clin Invest 2003;112:575–87). We have produced mAbs to a Cripto 17-mer peptide within the EGF-like region of Cripto and selected mAbs by using growth inhibition rather than by conventional serology. We report here that (a) mAbs to Cripto react with a number of cancers but do not react or react weakly with normal tissues; (b) the Cripto mAbs inhibit cancer cell growth in vitro and inhibit established tumor growth of colon cancer xenograft in Scid mice; and (c) the mAbs inhibit Cripto Akt signaling and activate stress-activated protein kinase (SAPK)c-Jun NH2-terminal kinase (JNK) and p38 pathways, leading to apoptosis, demonstrated by propidium iodide (PI), DNA fragmentation, and poly(ADP-ribose) polymerase (PARP) cleavage assays. These results indicate that targeting Cripto using mAb can interrupt Cripto function and may be a useful approach to cancer therapy.

MATERIALS AND METHODS

Production and Testing of mAbs. A 17-mer peptide from amino acids 97–113 (CPPSFYGRNCEHHDYRKE) of Cripto was synthesized using an Applied Biosystems Model 430A automated peptide synthesizer (Foster City, CA) and conjugated to keyhole limpet hemocyanin (KLH); 100 μg Cripto 17-mer-KLH, emulsified in complete Freund’s adjuvant, were injected i.p. into Lewis female rats. After 4 weeks, a second injection of Cripto 17-mer-KLH was given, and 3 days after a third injection, the spleen cells were fused with murine myeloma NS1 cells (32, 33). The supernatants of hybridomas were determined using antirat immunoglobulin subclass antibodies (ICN, Irvine, CA). The absorbance (OD) value was measured at 405 nm in ELISA. Cripto 17-mer-KLH (5 μg/ml) or KLH (5 μg/ml) in carbonate buffer (pH 9.6) was coated onto the wells of a 96-well polystyrene plate (Costar, Cambridge, MA). Following blocking with 1% BSA, culture supernatants were added to the wells, and the binding of the antibodies was detected by sheep antirat immunoglobulin labeled with horseradish peroxidase (Dako A/S, Glostrup, Denmark). The absorbance (OD) value was measured at 405 nm after adding the substrate 0.03% 2,2-azino-di-3-ethylbenzthiazoline sulfonate/0.02% H2O2 (33).

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Cell Lines. Cancer cell lines used were LS174T and HT-29 (colon); MCF-7, MDA231, and ZR75 (breast); DU145, PC3, and LNCap (prostate); and Ben and Colo383 (lung). CCRI-CRM and its drug-resistant variant CEMA7R (leukemia; Ref. 34), and the human embryonic cell line car. Cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS at 37°C in a 5% CO2 humidified incubator.

Flow Cytometry. Cells (2 × 10^6) were incubated with mAb for 30 min at 4°C, and after washing with PBS containing 2% newborn calf serum, FITC-conjugated sheep F(ab)2, antirat immunoglobulin (Dako) was added; after washing, the cells were resuspended in PBS and examined with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ; Ref. 33). To measure cell death, PI was added to the cells after incubation of the cells with anti-Cripto mAbs.

Immunoperoxidase Staining. Human tissues were obtained from the Department of Anatomical Pathology, Austin and Repatriation Medical Centre, Australia, and stained with the anti-Cripto mAbs using the immunoperoxidase technique on formalin-fixed tumor tissues using antirat immunoglobulins linked to horseradish peroxidase (Dako; Ref. 33).

Cell Proliferation Assays. First, for [3H]thymidine incorporation, 5 × 10^3 cells/well were incubated in 96-well plates with various concentrations of anti-Cripto mAbs, negative control mAb BCP7 (antihuman MUC1 peptide), or cytotoxic agents epirubicin, cisplatin, or 5-fluorouracil (5-FU). After 48 or 72 h, the cells were pulsed with [3H]thymidine (1 μCi/ml) for 4 h. [3H]thymidine incorporation was measured by TopCount (Perkin-Elmer, Boston, MA). All of the assays were performed in triplicate, and the results were expressed as percentage of incorporation of [3H]thymidine in the treated group to controls (medium only) (34). Second, to measure viable cell numbers, the trypan blue dye exclusion method was used. Cells were seeded in 24-well flasks at an initial concentration of 5 × 10^4 cells in 10 ml of medium and incubated overnight; anti-Cripto mAbs (30 μg/ml) then were added to the culture. Five days later, viable cells were counted (36). For CCRI-CRM and CEMA7R, 2 × 10^6 cells were incubated with C4 or C13, and viable cells were counted on day 3 (34, 36).

Antitumor Effect in Vivo. Scid mice were inoculated s.c. with human colon cancer cell line LS174T cells (2.5 × 10^6). Eight hours later, the mice received 0.5 mg C13, followed by another 0.25 mg i.p. as indicated in Fig. 3. Tumors were measured at 2- to 4-day intervals with calipers, and tumor size was calculated (length × width × height; Ref. 37). To further test antitumor efficacy of the Cripto mAb, an established LS174T tumor in Scid mice was treated with C13 (0.5 mg) i.p. on day 11 when the tumors had reached an average size of 225 mm^3, followed by 0.25 mg C13 as indicated in Fig. 3.

DNA Fragmentation. Cancer cells (1 × 10^6) were treated with anti-Cripto mAbs for 72 h and lysed in 10 mM EDTA, 10 mM Tris (pH 8.0), and 0.5% Triton X-100 on ice for 15 min. After centrifugation, soluble (fragmented) DNA was separated from the pellet and treated with RNase A (50 μg/ml) and proteinase K (100 μg/ml) in 0.5% SDS at 50°C for 2 h. The fragmented DNA was extracted with phenol/chloroform, precipitated in ethanol, electrophoresed on a 2% agarose gel, and stained with ethidium bromide (38).

Western Blot and Signal Transduction Analysis. Colon cancer LS174T cells (5 × 10^6) were treated with mAbs for various times. The cells were lysed and sonicated in 0.5 ml lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium PPr, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenethyl alcohol (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were blocked with 5% nonfat dry milk in Tris-buffered saline buffer with 0.1% Tween-20 at room temperature for 1 h and probed with appropriate dilution of anti-Cripto mAb C4. To measure the signaling pathways, anti-phospho p44/p42 MAPK (Thr202/Tyr204; New England Biolabs, Beverly, MA), anti-phospho-Akt (Ser473), and anti-phospho p38 SAPK (Thr180/Tyr182; Promega, Madison, WI) antibodies were used. An anti-PARP (PharMingen, San Diego, CA) was also used to detect fragment (M, 85,000) of PARP. The proteins in the polyvinylidene difluoride membranes were visualized using chemiluminescence reagent (Perkin-Elmer) after adding horseradish peroxidase-labeled secondary antibodies. The JNK activity was measured using an SAPK/JNK assay kit (New England Biolabs). A recombinant fusion protein c-Jun residue 1–89 and glutathione S-transferase (GST-c-Jun) were used as the substrate for activated JNK. Briefly, the supernatant of cell lysate was incubated with immobilized GST-c-Jun fusion protein overnight at 4°C to precipitate activated JNK.

Kinase reaction was carried out in vitro at 30°C for 30 min in kinase reaction buffer [25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, and 10 μM MgCl2] containing 100 μM ATP. Phosphorylation of GST-c-Jun on Ser-63 was analyzed by immunoblotting using anti-phospho-specific c-Jun (Ser-63) antibody as described previously.

Statistical Analyses. Statistical analyses were performed using GraphPad InStat software (GraphPad Software Inc., San Diego, CA). The Mann-Whitney nonparametric U test was used to compare the tumor sizes in groups of mice treated with mAb and PBS, respectively.

RESULTS

Characterization of mAbs to Cripto. After fusion of rat spleen cells and murine myeloma cells, hybridomas were screened by ELISA; those reacting with the Cripto 17-mer peptide but not with carrier KLH were screened in a cell growth assay to select mAbs that gave >60% of inhibition of [3H]thymidine incorporation by the Cripto-positive breast cancer MCF7 and colon cancer LS174T cell lines. Of 17 rat mAbs produced, 10 of 17 gave inhibition, of which 2 mAbs (C4 and C13, both IgM) are reported here. The reaction of the mAbs was specific for Cripto because they reacted with the 17-mer peptide and with a 38-mer peptide (corresponding to 76–113 amino acids of Cripto EGF-like region and containing the 17-mer peptide; data not shown) but not with KLH or other peptides (Fig. 1A).

In addition, by Western blot analysis the mAbs reacted with M, 36,000 molecule from lysates of cancer cell lines DU145, LNCap, MCF7, and LS174T but not from Cripto-negative human 293 cells (Fig. 1B). The immunoperoxidase staining of anti-Cripto monoclonal antibodies (mAbs). A. ELISA, showing reaction of mAb C13 tissue culture supernatant with keyhole limpet hemocyanin (KLH)-17-mer and KLH. B. Western blot analysis using C13 with lysates of human cancer cell lines of prostate (DU145 and LNCap), breast (MCF7), colon (LS174T), and the embryonal kidney cell line 293. C–H, immunoperoxidase staining of colon (C), breast (D), and lung (E) cancers and normal colon (F), breast (G), and lung (H) tissues with mAb C4 showing cell surface and cytoplasmatic staining of cancer cells (brown), and no staining in normal tissues. I–K, immunoperoxidase staining of breast cancer cell line MCF7 with C4 (I) and C13 (J), and immunofluorescence staining of MCF7 with C4 (K).

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M_36,000 band also was identified using a rabbit anti-Cripto 17-mer antibody (data not shown).

**Tissue Distribution of the mAbs.** By immunohistochemistry the C4 mAb reacted with formalin-fixed cells [cancers of the colon (7 of 9), breast (5 of 7), lung (18 of 20), stomach (3 of 4), and pancreas (2 of 3)] but did not react or reacted weakly with normal colon (0 of 8), breast (2 of 4), lung (0 of 4), stomach (0 of 2), pancreas (1 of 2), and liver (2 of 2) cells (Fig. 1, C–H). In reactive cells, the staining was on the cell surface and in the cytoplasm (Fig. 1, C–E). Cell lines MCF-7 and LS174T also were reactive by immunoperoxidase and immunofluorescence staining with strong staining of the cell surface and cytoplasm (Fig. 1, I–K). The results indicate that Cripto is highly expressed in most malignant tumors and cancer cell lines but is absent or weakly present in normal tissues.

**Anti-Cripto mAbs Inhibited the Growth of Cancer Cells.** C4 and C13 mAbs inhibited in vitro the growth of cancer cell lines HT29 and LS174T (colon cancers); MCF7, MDA-231, and ZR-75 (breast cancers); DU145 and PC3 (prostate cancers); Colo 338 and Ben (lung cancers); and CCRF-CEM (leukemia) and its drug-resistant variant CEM/A7R when tested by [^{3}H]thymidine incorporation. Inhibition of 70–95% of thymidine incorporation was observed in most tested cancer cell lines (Fig. 2A). The inhibition of mAbs also was examined by counting cell numbers using trypan blue exclusion (Fig. 2B). There was no inhibition on the growth of the Cripto-negative 293 cells (Fig. 2, A and B), and control nonreactive mAb had no effect on the growth of either tumor cells or 293 cells (not shown).

**Combination of mAbs with Cytotoxic Drugs in Vitro.** Anti-HER-2/neu antibodies such as Herceptin can have increased activity in combination with cytotoxic drugs (37), and this also was demonstrated by the anti-Cripto mAbs with three different drugs (Fig. 2, C–E). For example, C4 (10 µg/ml) inhibited growth of LS174T by 26.4% and 5-FU (1.5 µg/ml) by 21.6%; the combination of C4 and 5-FU inhibited growth by 52.2% (Fig. 2C). The enhanced cytotoxicity also was demonstrated at different concentrations of C4 (10–30 µg/ml) and 5-FU (1.5–3.0 µg/ml; Fig. 2C) and in the combination of C4 with epirubicin or cisplatin on LS174T cells (Fig. 2, D and E). Similar results also were obtained on MCF7 (data not shown). Thus, anti-Cripto-1 mAbs could inhibit cell growth in vitro, and the combination of the mAbs and cytotoxic drugs enhanced the inhibitory effects.

**Cancer Growth Inhibition of Colon Cancer in Mice.** The antitumor effect of Cripto mAb in vivo was demonstrated by two xenograft models. First, in the preventive model, Scid mice were treated with C13 8 h after the inoculation of LS174T cells. Each mouse received six injections (total of 1.75 mg) of C13 mAb. Tumors in mice treated with C13 were eradicated completely (Fig. 3A). Second, in the...
therapeutic model, to confirm the inhibitory effect of the mAbs, Scid mice with established tumors (average size $225 \text{ mm}^3$) were treated with C13 (0.5 mg) on day 11, followed by another four injections (total of 1.5 mg). The tumor size was reduced significantly (80%) in the C13-treated group ($540 \text{ mm}^3$) compared with untreated control ($2650 \text{ mm}^3$; $n = 6$; $P < 0.05$; Fig. 3B). Thus, C13 can inhibit cancer cell growth not only in vitro but also in vivo.

Apoptosis Induced by Cripto mAbs. Because mAbs decreased $[^{3} \text{H}]$thymidine uptake (Fig. 2A) and absolute cell numbers (Fig. 2B), it was likely that there was apoptosis, and this was shown by three assays: (a) the presence of dead cells could be shown by PI staining: 80% dead cells were obtained with C4 compared with 15% with a negative control mAb (Fig. 4A); (b) DNA fragmentation of LS174T cells occurred after 72-h incubation with C4 (Fig. 4B); and (c) the production of an $M_s 85,000$ fragment of PARP (an $M_s 116,000$ nuclear PARP) was shown by Western blot analysis after treatment of LS174T with C4 (Fig. 4C). DNA fragmentation and the cleavage of PARP (appearance of the $M_s 85,000$ fragment) indicate activation of the downstream effector caspase-3.

Signal Transduction Induced by Cripto mAbs. Cripto is reported to activate MAPK (ras/raf/MAP/ERK kinase/MAPK) and phosphatidylinositol 3'-kinase/Akt (26, 27). However, the effect of Cripto on SAPK/JNK and p38, members of MAPK family, has not been reported. The effect of anti-Cripto mAbs on the activation of Akt, MAP/ERK kinase/MAPK, and proapoptotic signaling pathways of SAPK/JNK and p38 was examined.

Effect of Anti-Cripto mAbs on Akt and MAPK Activation. Akt is activated by various growth factors through phosphorylation of Thr308 and Ser473 at the COOH-terminus to promote cell survival and proliferation. With C4 treatment (5 or 10 $\mu$g/ml), the level of Akt phosphorylation in LS174T cells was decreased at 3 h (Fig. 5A); by 8 h, the level had returned to normal (Fig. 5B and C) possibly because of the secretion of soluble Cripto from LS174T cells (27). There was no change in the level of P44/42 (MAPK) after treatment of LS174T with C4 for 3 h (Fig. 5A), indicating the mAb was not involved in the MAPK pathway.

Activation of JNK and p38. JNK can be activated by stress (including UV and $\gamma$ radiation), inflammatory cytokines, and some growth factors and could be tested by GST-c-Jun phosphorylation. C4 activated JNK after 3-h treatment of LS174T cells (10 $\mu$g/ml $> 5$ $\mu$g/ml), indicated by phosphorylation of GST-c-Jun (Fig. 5A). The
highest level of activated JNK occurred after 24-h exposure to C4 (Fig. 5, B and C), least at 48 h, with a return to basal level by 72 h. C4 activates JNK in a dose- and time-dependent manner. The level of p38 in LS174T cells was increased by 24 h, further increased by 48 h, and reached the highest level at 72 h. The results show that C4 affects three separate signaling pathways: SAPK/JNK, p38 activation, and Akt inhibition, all of which are likely to be integrated into the apoptotic death signal. There was no effect on MAPK. We also noted that the expression of Cripto in LS174T cells was not changed after treatment with C4 for 3–72 h (Fig. 5); this differs from that obtained with Herceptin, which reduced cell surface HER-2/neu expression (39).

DISCUSSION

We now describe new mAbs to Cripto, which have an inhibitory effect on cancer cell growth in vitro and in vivo. The mAbs were produced to a Cripto 17-mer peptide, contained within the “EGF-like” region of Cripto (so called because of the position of the six cysteines in this domain; the overall protein sequence identity with EGF is ~25%). To generate inhibitory antibodies in the early screening procedures, the mAbs were selected using growth inhibition rather than ELISA or other serologic assays. Consequently, the mAbs reacted with Cripto and inhibited the growth of cancer cells in vitro and in vivo; in addition, they inhibited the growth of an established human colon cancer xenograft in Scid mice. The in vitro antitumor activity of the anti-Cripto mAbs was increased when combined with cytotoxic drugs such as 5-FU, epirubicin, and cisplatin, suggesting that adjuvant therapy with selected drugs in patients could include these Cripto mAbs to enhance the therapeutic effect. We note that Cripto antisense oligonucleotides were reported to inhibit the proliferation of tumor cells (40, 41). Other anti-Cripto mAbs more recently have been described that have growth inhibitory activity (31). These mAbs to the Cripto CFC domain suppressed tumor cell growth in vitro and in vivo (31). Thus, study by Adkin et al. differs from ours in several important aspects: (a) our mAbs react with the Cripto-EGF-like domain rather than with the CFC domain; (b) the mAb in the study by Adkins et al. disrupted Cripto-Nodal signaling, whereas our mAbs affect MAPK/JNK, P38, and Akt signaling; and (c) our mAbs are IgM and could more efficiently cross-link Cripto. However, the possibility of two different signaling sites is of interest and potential therapeutic value because the two mAbs together could be more potent.

In addition to the inhibitory activity, Cripto is a useful target for immunotherapy because of its unique tissue distribution. Cripto is present at low levels in some normal tissues but is highly expressed on many cancers (60–90%) by immunoperoxidase staining and mRNA expression (18–23) compared with EGF and its receptors (the most studied in this field), which are expressed in more normal tissues and in fewer cancers (30–50%) than Cripto (39, 42–44).

We demonstrate that there were several different mechanisms for the inhibition of cell growth. First, apoptosis occurred, demonstrated by PI staining, DNA fragmentation, and PARP cleavage (Fig. 4), which indicated that activation of nuclease and caspase-3, a downstream effector of caspase family. Second, another mechanism of inhibition of cell growth was observed caused by the sustained activation of p38 and MAPK/JNK, which led to cancer cell apoptosis. The activation of p38 and JNK is unique and is not seen with other therapeutic mAbs, such as Herceptin and Rituximab (anti-CD20 mAb) unless they are cross-linked by a second antibody (45, 46). Apart from activation of p38 and MAPK/JNK, there was inhibition of Akt phosphorylation by anti-Cripto mAbs. Akt phosphorylation is a crucial step for cell growth and survival, and its inhibition could lead to apoptosis (27). Similar inhibition also is found with the HER-2/neu mAb Herceptin (47, 48). Although the inhibitory effect on Akt phosphorylation by Cripto mAb was transient, it may be important to initiate the process of apoptosis. Thus, Cripto-induced apoptosis may be the outcome of the balance of inactivation/activation of a number of signal transduction pathways affected by the anti-Cripto mAbs (49).

To initiate their actions, Cripto mAbs could bind to either soluble Cripto (ligand) or cell surface bound Cripto (coreceptor) or to both forms simultaneously to interfere with Cripto function. Other studies have shown that Cripto peptide p47-mer (corresponding to residues 67–113 spanning the EGF-like region of Cripto) can bind to Glypican-1 and activate c-Src to induce MAPK and Akt activation (24, 26, 48), cell migration (16), and branching (13), indicating the p47-mer in the EGF-like domain is a ligand/receptor-interacting motif. Furthermore, the EGF-like domain also is required for Cripto to bind activin in the presence of type II activin receptors to block activin signaling as an additional mechanism for cell growth (28). Our results indicate that the 17-mer motif included in the 47-mer may be the crucial motif for Cripto function. Therefore, the mAbs to the 17-mer peptide may affect Cripto function in four ways: (a) Cripto, a glycosyl-phosphatidylinositol-anchored protein, which forms rafts, could be cross-linked by Cripto mAbs, resulting in activation of the associated Src family protein tyrosine kinases (48); (b) the mAbs bind to cell surface Cripto and trigger MAPK/JNK and p38 signaling transduction; (c) the mAbs block the binding of soluble Cripto to Glypican-1 and inhibit activation of Akt; and (d) the mAbs could block the binding of Cripto to activin and abolish antagonism of Cripto on activin signaling pathways, resulting inhibition of cell growth. To determine whether the antibodies react with cell surface and/or soluble Cripto and which of these mechanisms is important is the subject of current studies.

The results indicate that Cripto is a unique target, and mAbs to Cripto have a potential to manage various cancers. Therefore, it is appropriate to develop humanized mAbs to Cripto for clinical trial; these could be more effective than the rat mAbs by activation of complement to lead to opsonization and cell lysis or involve Fc receptors to lead to antibody-dependent cellular cytotoxicity in addition to apoptosis induced through signal transduction (50).

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