CD73 is a cell surface 5′-nucleotidase that converts AMP to adenosine, an immune suppressive molecule. CD73 may promote immune escape in cancer by contributing to the degradation of extracellular ATP released by dying cancer cells in hypoxic tumors or following chemotherapy. However, whether CD73 exerts a critical oncogenic function during tumorigenesis is unknown. In this study, we used genetically deficient mice to investigate its contribution to autochthonous tumor formation. CD73 deficiency suppressed the development of 3-methylcholanthrene (MCA)-induced fibrosarcomas through a mechanism relying upon IFN-γ, natural killer (NK) cells, and CD8+ T cells. Similarly, CD73 deficiency also suppressed prostate tumorigenesis in TRAMP transgenic mice. Importantly, treatment with an anti-CD73 monoclonal antibody effectively suppressed growth of established MCA-induced tumors or TRAMP-C1 prostate tumors and inhibited the development of TRAMP-C1 lung metastases. The therapeutic activity of anti-CD73 monoclonal antibody against primary tumors was dependent on CD8+ T cells, whereas its antimitastatic activity was dependent on host CD73 expression independent of T cells or NK cells. Taken together, our findings indicate that CD73 is a critical factor in tumorigenesis and that anti-CD73 antibodies may offer a novel generalized strategy to blunt immune escape and treat cancer. Cancer Res; 72(9): 2190–6. ©2012 AACR.
that arise spontaneously. Spontaneous de novo tumors develop over many weeks, which allows for the establishment of a unique tumor microenvironment similar to that seen in cancer patients. We here investigated the role of CD73 on de novo tumorigenesis.

Materials and Methods

Mice

Inbred C57BL/6 wild-type (WT) and CD73-deficient (CD73−/−) mice (kindly provided by Dr. Linda H. Thompson, Oklahoma Medical Research Foundation, Oklahoma) were bred and maintained at the Peter MacCallum Cancer Centre as previously described (15). C57BL/6 CD73-deficient TRAMP transgenic mice (CD73−/−/TRAMP) were generated by backcrossing C57BL/6 TRAMP with CD73−/− mice until CD73−/− TRAMP mice were generated. Female CD73−/− TRAMP mice were then bred with male CD73−/− mice and offspring screened for the TRAMP transgene as described below. All mice were routinely screened for viruses, parasites, and other microbes and tested negative over the entire course of the experiment. All experiments were carried out in accordance with guidelines set out by the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee.

Genetic screening of mice

DNA was extracted from the tails of 10- to 21-day-old litters of TRAMP mice and CD73−/− TRAMP mice using the Puregene DNA Purification Kit (Flowgen Bioscience Limited), as per their instructions. Presence of the TRAMP transgene was detected by PCR using an annealing temperature of 55°C. The sequences of the primers used for TRAMP detection were (5’ → 3’): 994, CCGTGGCAACGGGAAGCTCTCCACAAAGTGCAATATA and 995, CTCCTTCTCAAGCCTAGGATCTCA, which produced a 600 bp PCR fragment from TRAMP tg DNA. Primer sequences to ensure integrity of the PCR reaction were (5’ → 3’): 992, GATGTTGCTCCAGGCTAAAGTT and 993, AGAAACGGAATGTTGTAGGTAGGT, which generated a PCR product of 500 bp. Presence of the CD73 mutant allele was detected by PCR using an annealing temperature of 55°C. The sequences of the primers used for wild-type CD73 detection were (5’ → 3’): 2062, TCTTTAGGGACAAATTTAGTC and 2063, AGAAAGGTGGTTGGAGTGCCT, which produced a 188 bp PCR fragment from DNA. Primer sequences for the mutant CD73 allele (neo) were (5’ → 3’): 2060, CTTGGGTGGAGAGGCAGTGTTT and 2061, AGGGTGAGTTGGAGGAGATC, which generated a PCR product of 280 bp.

Cell lines and antibodies

TRAMP-C1 mouse tumor cell lines were maintained as previously described (18). MCA.WT100-5 and MCA.WT100-7 cells were derived from WT mice that had been inoculated with 100 μg of 3-methylcholanthrene (MCA). CD73 expression was assessed using phycoerythrin (PE)-conjugated anti-mouse CD73 mAb (clone TY2/3; BD Bioscience). PE-conjugated anti-mouse CD45.1 (A20), fluorescein isothiocyanate (FITC)-conjugated anti-mouse Gr1 (RB6–8C5), Pacific Blue-conjugated anti-mouse CD4 (L3T4), PE-conjugated anti-mouse CD25, allophycocyanin (APC)-conjugated anti-mouse CD8 (53–6.7), were purchased from BD Bioscience, FITC-conjugated anti-mouse CD11b (M1/70), FITC-conjugated anti-mouse Foxp3, and (APC)-conjugated anti-mouse CD45.2 (104) were purchased from eBioscience. Purified anti-CD73 mAb (clone TY/23; provided by Dr. Linda H. Thompson, Oklahoma Medical Research Foundation, Oklahoma), anti-CD4 mAb (GK1.5), anti-CD8 mAb (53.6.7), and control Ig (clg; clone MAC4) were produced in house.

MCA-induced fibrosarcoma

Groups of 15 to 20 male WT or CD73−/− mice were inoculated s.c. in the hind flank with 25, 100, or 400 μg of MCA (Sigma-Aldrich) in 0.1 mL of corn oil as described (19). Some WT mice received clg or weekly depletion/neutralization of CD8+ T cells (53.6.7), NK cells [anti-asialoGM1 (ASGM1)], or IFN-γ (H22, kindly provided by Dr. Schreiber, St Louis, MO) from the time of MCA inoculation to day 56 (100 μg i.p.). Development of fibrosarcomas was monitored weekly over the course of 300 days. Measurements were made with a caliper square as the product of 2 perpendicular diameters (mm²) and individual mice are represented. For percent survival tumors more than 100 mm² in size were recorded as lethal.

TRAMP transgenic mice

CD73+/+ TRAMP and CD73−/− TRAMP mice were monitored twice weekly and when the abdomen became distended, the age of the mouse recorded and a postmortem conducted. Mean prostate weight ± SEM at various ages (13, 20, and 25 weeks) were calculated and probability of significance determined using a Mann–Whitney rank-sum U test.

Immunofluorescence

Prostates were harvested from 20-week-old CD73+/+ TRAMP and CD73−/− TRAMP mice, frozen in OCT media, sectioned and stored at −80°C. For immunofluorescence staining, sections were thawed at room temperature before fixation in ice-cold acetone for 10 minutes. Sections were then washed in PBS and then incubated with anti-CD4 (RM4-5; BD Pharmingen), anti-CD8 (53–6.7; BD Pharmingen), or anti-CD73 (TY/23) diluted 1 in 200 in DAKO antibody diluent. After 90 minutes, sections were washed in PBS and then probed with Alexa Fluor 488 conjugated goat anti-rat (Invitrogen-A11006) diluted 1 in 1,000. Sections were incubated for 75 minutes, washed in PBS, and then mounted with Prolong Gold plus DAPI (Invitrogen). CD4/CD8 cells were quantified by recording the average number of cells within a section and 3 sections per prostate using a BX-51 Olympus microscope.

Subcutaneous tumors

TRAMP-C1 tumor cells were injected subcutaneously into syngeneic C57BL/6 wild-type (WT) or C57BL/6 CD73−/− mice at the indicated doses. Where indicated, mice were treated with clg or anti-CD73 mAb (TY2/3).
Metastatic tumor models

To examine metastatic tumor growth, WT or CD73−/− mice were inoculated intravenously (i.v.) with increasing doses of TRAMP-C1 prostate carcinoma cells. Mice were monitored, harvested, and lung metastases quantified as previously described (15). Some mice were treated with cIg (Mac-4) or anti–IFN-γ, anti-CD8β (53.5.8) and anti-CD4 (GK1.5) or anti-asialoGM1 (Wako Chemicals; as indicated in the legends) to neutralize IFN-γ or deplete cell subsets.

Tumor-infiltrating lymphocytes

MCA-induced fibrosarcomas were excised, minced with scissors, and incubated 1 hour at 37°C for in PBS containing collagenase type 4 (Worthington Biochemical) and DNase I (Roche). Tumor cell suspensions were passed through a 70-μm cell strainer, washed twice in PBS, and resuspended in PBS-containing 2% serum for flow cytometry analysis. Anti-CD16/32 mAb (clone 2.4G2) was used to block Fc receptors. Flow cytometry was carried out on an LSR II (BD Bioscience) and analyzed using the software program FCS Express.

Statistics

Statistical analyses were conducted using Graph Pad Prism software. Significant differences in tumor growth and metastases were determined by a Mann–Whitney test. Statistical differences in % mice survival were determined by a Log-rank Mantel-Cox test. P < 0.05 was considered significant.

Results and Discussion

CD73-deficient mice are resistant to MCA-induced tumor initiation

We assessed the importance of CD73 in the MCA induction model of fibrosarcoma, in which host immunity plays a critical factor in suppressing tumor initiation and progression. As shown in Fig. 1A, host CD73 deficiency caused a significant reduction in the induction of fibrosarcomas by MCA. Tumor incidence in CD73-deficient mice was significantly reduced at all doses of MCA tested (Supplementary Fig. S1A–S1B). We also assessed the effect of CD73 deficiency on the growth rates of tumors that developed in CD73-deficient and WT mice. We observed that tumor growth rates were significantly suppressed in CD73-deficient compared with WT mice (Fig. 1B). We next determined the effect of CD73 deficiency on immunosurveillance of MCA-induced tumors. For this purpose, we compared tumor initiation in WT (Fig. 1C) and CD73-deficient mice (Fig. 1D) depleted of NK cells and/or CD8+ T cells, or treated with a neutralizing anti–IFN-γ mAb from the time of MCA inoculation. Our results revealed that depletion of NK cells and IFN-γ abolished the protective...
The effect of CD73 deficiency on MCA-induced tumor initiation, while depletion of CD8+ T cells did not reach statistical difference (Fig. 1D).

**CD73 inhibits adaptive immunity in established MCA tumors and can be targeted for treatment**

We next investigated the role of CD73 on the immunosurveillance of established MCA-induced tumors. In these experiments, mice injected with MCA were depleted of NK cells and/or CD8+ T cells after tumor initiation (i.e., from palpable tumor formation). As shown in Fig. 2A, depletion of CD8+ T cells, but not NK cells, after tumor initiation completely abolished the protective effect of CD73 deficiency on tumor growth rates. We next assessed whether targeted blockade of CD73 could effectively delay growth rate of established MCA-induced tumors. Groups of mice were inoculated with MCA and treated with biweekly injections of a cIg or anti-CD73 mAb starting at tumor onset. As shown in Fig. 2B, treatment with anti-CD73 mAb significantly suppressed the growth of established MCA-induced tumors. Individual growth curves of treated mice are shown as Supplementary Data (Supplementary Fig. S1C–S1D).
Tumor and host CD73 are targeted by anti-CD73 mAb treatment

We next investigated whether CD73 was expressed on MCA-induced tumor cells. Tumor cell lines were established from WT mice inoculated with MCA and analyzed by flow cytometry for CD73 expression. CD73 was heterogeneously expressed amongst different MCA fibrosarcoma cell lines. The majority of MCA tumor cell lines were negative or expressed low levels of CD73 (Supplementary Fig. S2). We also investigated whether CD73 was expressed on various tumor-infiltrating immune cells. Consistent with previous studies (14–16), CD73 was expressed on tumor-infiltrating CD8\(^+\) T cells, CD4\(^+\) T cells, and Foxp3\(^+\) T regulatory cells, but absent on tumor-infiltrating CD11b\(^+\) or CD11c\(^+\) cells (Supplementary Fig. S3). We next sought to determine the role of tumor and host CD73 on anti-CD73 mAb therapy of MCA-induced tumors. For this purpose, we compared treatment activity of anti-CD73 mAb against CD73\(^+\) and CD73\(^{-}\) MCA tumors in WT and CD73\(^{-}\) mice. Treatment of CD73\(^+\) tumors was effective in both WT and CD73\(^{-}\) mice (Fig. 2C), while treatment of CD73\(^{-}\) tumors was only effective in WT mice (Fig. 2D). Taken together, our results suggest that treatment of MCA tumors is mediated by targeting both tumor and host CD73.

CD73 promotes prostate cancer in TRAMP transgenic mice

We next assessed the role of CD73 in the development of de novo prostate tumors in TRAMP transgenic mice. Previous studies suggested that CD73 is associated with malignant transformation of prostate epithelial cells (20). TRAMP transgenic mice develop mild to severe prostate hyperplasia by 12 weeks of age and by 24 weeks of age, approximately 100% of male mice have poorly differentiated and invasive adenocarcinomas (18). We generated CD73\(^{-}\) TRAMP transgenic mice and compared prostate weights with CD73\(^+\) TRAMP mice. As shown in Fig. 3A, CD73 deficiency in TRAMP mice was associated with a significant reduction in prostate weights as early as 13 weeks of age. Significant reduction in prostate weights was also observed at 20 and 25 weeks of age. Immunohistochemistry revealed that TRAMP prostate tumors expressed CD73 (Supplementary Fig. S4). We next assessed the effect of CD73 deficiency on TRAMP tumor-infiltrating lymphocytes. As shown in Fig. 3B, CD73 deficiency was associated with an increased ratio of tumor-infiltrating CD8\(^+\) to CD4\(^+\) T cells (Fig. 3C).

Anti-CD73 mAb therapy inhibits TRAMP-C1 tumor growth and metastasis

We next investigated the role of host CD73 in the progression and metastasis of prostate cancer by inoculating WT and CD73\(^{-}\) mice with TRAMP-C1 tumors cells. The growth of primary TRAMP-C1 tumors was significantly, albeit modestly, delayed in CD73\(^{-}\) mice (Fig. 4A) in a CD8\(^+\) T cell-dependent manner (Supplementary Fig. S5). Remarkably, CD73\(^{-}\) mice were greatly resistant to experimental TRAMP-C1 lung metastases (Fig. 4B). On the basis of these observations, we next assessed the therapeutic activity of anti-CD73 mAb against
As shown in Fig. 4C, anti-CD73 mAb therapy significantly delayed the growth of primary TRAMP-C1 prostate tumors in a CD8⁺ T cell–dependent manner. We next assessed the antimitastatic activity of anti-CD73 mAb therapy. As shown in Fig. 4D, anti-CD73 mAb therapy significantly suppressed lung metastasis of TRAMP-C1 prostate tumors.
prostate cancer cells. Notably, this antimitastatic activity was dependent on host CD73 expression, independently of T cells or NK cells (Fig. 4D).

In conclusion, we showed that CD73 promotes carcinogen-induced tumor initiation and tumor growth by suppressing immunosurveillance via IFN-γ, NK cells, and CD8+ T cells. We also showed that CD73 promotes de novo prostate tumorigenesis. Furthermore, we provided the first evidence that anti-CD73 mAb therapy can suppress prostate tumor growth and metastasis. Our findings might have significant impact for prostate cancer treatment. The recent successes of cancer immunotherapies such as Sipuleucel-T (Provenge, Dendreon Corporation) and ipilimumab (Yervoy, Bristol Meyers Squibb) are paving the way for new immune-based treatments. Promising approaches include antagonists of immune checkpoint inhibitors, such as anti–PD-1 mAb, and immune-activating anti-CD137 mAb (21). Our study suggests that immunotherapy of prostate cancer may be significantly improved by combining these approaches with targeted blockade of CD73.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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