学 位 論 文 の 要 旨

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学 位 論 文 名　Immunogenic Chemotherapy with Cyclophosphamide and Doxorubicin against Established Murine Carcinoma

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論 文 内 容 の 要 旨

INTRODUCTION

Recent advances in tumor immunology have discovered crucial mechanisms that must be overcome for optimal anti-tumor immune activity *in vivo*. First, tumor-bearing states typically involve several types of immunosuppression via immunosuppressive cells such as $CD4^+$ $CD25^+$ regulatory T cells (Treg) and/or myeloid-derived suppressor cells. Specifically, Tregs have received a great deal of attention as suppressive cells in tumor-bearing patients. Although several methods such as treatment with antibodies can relieve Treg-mediated immunosuppression, a number of reports have revealed that cyclophosphamide (CP) can mitigate Treg-mediated immunosuppression when administered at a low dose. Second, presentation of tumor-derived antigens by dendritic cells (DCs) to T cells is a critical step for *in vivo* elicitation of anti-tumor T cell immunity, and some anti-cancer drugs such as anthracylines are known to exploit this process. In anthracycline-treated dying tumor cells, calreticulin, which is constitutively expressed in the endoplasmic reticulum, migrates to the cell surface, provides phagocytic signals to DCs, and consequently promotes their up-take. This immunogenic tumor cell death is crucial for treatment-associated prognoses and for the survival of tumor-bearing hosts. In this study, we investigated the potential synergistic activity of the combined use of CP and doxorubicin (DR), as an anthracycline drug, against established murine carcinoma.

MATERIALS AND METHODS

CT-26 and RENCA are colon carcinoma and renal cell carcinoma cell lines of BALB/c

mouse origin, respectively. BALB/c mice were injected subcutaneously (s.c.) with 2×10^5 CT-26 cells into the right flank and with 2×10^5 CT-26 cells or 2×10^5 RENCA cells into the left flank. On day 10, the mice received an intraperitoneal (i.p.) injection of CP at a dose of 100 mg/kg. On days 12, 14, and 16, the mice were injected intratumorally (i.t.) into the right-side tumor with either 120 μg of DR or 100 μg of mitomycin-C at a volume of 40 μl. After tumor inoculation, tumor size (mm^2) was measured twice weekly.

Total RNA was isolated from tumors, and the synthesized first-strand cDNA was amplified. Real-time PCR was carried out in duplicate using the ABI PRISM 7000 sequence Detection System. Relative mRNA levels as compared with β-actin were calculated.

To test specific T cell responses against a CT-26-associated tumor antigen, an H-$2L^d$-binding AH1 peptide (SPSYVYHQF), derived from the envelope protein (gp70) of an endogenous murine leukemia virus, was used. Tumor-draining lymph node cells were harvested, pooled, and stimulated *in vitro* with the indicated peptides. The levels of IFN-γ in the culture supernatants and the frequency of IFN-γ -producing cells were determined by enzyme-linked immunosorbent assay (ELISA) and the enzyme-linked immunosorbent spot assay (ELISPOT), respectively. To examine an effect of CP and/or DR on $CD4^+$ Treg, purified $CD4^+$ T cells from the tumor-bearing mice that were treated with or without CP and/or DR were cultured with whole naïve lymph node cells. The cell proliferation was assayed with standard thymidine ([methyl-^{3}H]TdR) incorporation and scintillation counting.

To compare protective anti-tumor activity induced by vaccination with either DR-treated or MMC-treated CT-26 cells, BALB/c mice were injected s.c. with 2×10^5 CT-26 cells into the right flank and simultaneously vaccinated s.c. with DR-treated or MMC-treated 1×10^6 CT-26 cells into the left flank. To inactivate tumor cells, CT-26 cells were cultured *in vitro* with DR (20 μg/ml) or MMC (10 μg/ml) for 24 h. As a protective model, BALB/c mice were vaccinated s.c. into the left flank with DR-treated 1×10^6 CT-26 cells that were pre-transfected with siRNA. On day 14, the mice were inoculated s.c. with 2×10^5 CT-26 cells into the right flank.

RESULTS AND DISCUSSION

We first determined whether combination therapy with CP and DR could show synergistic anti-tumor activity against s.c. established CT-26 colon carcinoma. The i.p. injection of CP significantly suppressed bilateral CT-26 growth. I.t. injection of DR led to a slight suppression in growth of the CT-26 cells on the DR-treated side; no effect on remote CT-26 cells was observed. Combination therapy significantly suppressed growth of the CT-26 cells on the DR-treated side, and the growth of remote CT-26 cells was also suppressed by approximately 50% compared to CP alone. In contrast, combination therapy failed to show any synergistic effect on remote RENCA carcinoma. We next combined CP treatment with another cytotoxic drug, MMC, but no synergistic effect with CP was observed. In addition, no synergistic effect of CP and DR was observed when CT-26 cells were inoculated into BALB/c nu/nu mice.

We next attempted to detect tumor-specific T cell responses in CT-26-bearing mice that were treated with combination therapy. As a result, AH1 peptide-specific IFN-γ production was observed only in tumor-draining lymph node cells from mice treated with combination therapy. We also performed an ELISPOT assay to examine the frequency of AH1 peptide-specific T cells. The tumor-draining lymph node cells from the combination treatment group produced a higher level of IFN-γ in response to the AH1 peptide compared with other groups.

We next compared an immunosuppressive activity of tumor-draining lymph node cells from the mice that were treated with or without CP and/or DR. As a result, CP treatment significantly relieved immunosuppressive capacity of $CD4^+$ T cells in the tumor-draining lymph node cells, and the CP-induced restoration of immune reactivity was significantly augmented when combined with DR treatment. In addition, real-time PCR revealed that the combination therapy significantly increased the mRNA expression of IFN-γ and TNF-α and suppressed the expression of Foxp3 and TGF-β.

DR is reported to induce immunogenic tumor cell death through the surface expression of calreticulin, which provides 'eat-me' signals to DCs. Therefore, we tested the participation of calreticulin in the anti-tumor effects induced by combination therapy. Athough all seven mice rejected the CT-26 challenge when they were pre-vaccinated with control siRNA-transfected and DR-treated CT-26 cells, three of seven mice failed to reject challenged CT-26 when they were pre-vaccinated with calreticulin siRNA-transfected and DR-treated CT-26 cells. These results suggest that calreticulin plays a partial role in anti-tumor immune activity induced by combination therapy with CP and DR.

Combination therapy with CP and DR led to anti-tumor activity not only against the DR-treated-side tumor, but also against the DR-untreated remote tumor. Because synergistic activities were not observed either when CT-26 cells and irrelevant RENCA were injected s.c. into each side or when BALB/c nude mice were used, the induced anti-tumor activity was tumor-specific and T-cell dependent. In addition, combination therapy significantly increased the frequency of tumor antigen-reactive and class I-restricted T cells in the tumor-draining lymph nodes. Furthermore, combination therapy tended to restore local production of IFN-γ and TNF-α and mitigated Treg-mediated immunosuppression within the tumor tissues. Overall, these results provide an immunological rationale for the combined use of the chemotherapeutic drugs, CP and DR.

CONCLUSION

Combined chemotherapy with CP and DR synergistically elicits tumor-specific T cell responses in tumor-bearing hosts. Because both drugs have been used clinically worldwide, this type of immunogenic chemotherapy could be easily combined with the use of current cancer vaccines.