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IFN-α-Expressing Tumor Cells Enhance Generation and Promote Survival of Tumor-Specific CTLs

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IFN-α gene therapy has been successfully applied in several tumor models. Our studies involving the murine colorectal adenocarcinoma cell line MC38 confirm that IFN-α transduction of a poorly immunogenic tumor cell reduces tumorigenicity and leads to long-lasting tumor immunity. To investigate the effect of IFN-α transduction on the development of antitumor immune responses, we restimulated splenocytes from MC38-immune mice in vitro. Detection of MC38-specific cytotoxicity was markedly enhanced when murine IFN-α2-transduced MC38 (MC38-IFNα) or CD80-transduced MC38 (MC38-CD80) was used for restimulation compared with wild type (MC38-WT) or neomycin resistance gene-transduced MC38 (MC38-Neo) cells. MC38-specific CD8+ CTL line and clone were established from splenocytes of mouse immunized with MC38-IFNα. Stimulation with MC38-IFNα as well as MC38-CD80 enhanced the proliferation of MC38-specific CTLs in vitro much more effectively than stimulation with WT or MC38-Neo (p < 0.05). Coincubation of MC38-specific CTLs with MC38-IFNα or MC38-CD80 resulted in significantly less DNA fragmentation (8.0% and 12.8%, respectively) compared with coincubation of the CTLs with MC38-WT (43.5%; p < 0.001) or MC38-Neo cells (38.1%; p < 0.003). These results suggest that prevention of apoptotic cell death in tumor-specific CTLs may be one mechanism by which IFN-α-expressing tumor cells can promote the generation of antitumor immunity. The effect of IFN-α on CTLs appears to be similar to that of CD80, which also prevents apoptotic cell death after stimulation of T lymphocytes. The Journal of Immunology, 2000, 164: 567–572.

Interferon was first identified in 1957 as a factor that interferes with viral infection (1). Subsequent studies distinguished type I IFNs (later IFN-α and -β), which were produced in large amounts during the early phase of viral infection, and type II IFN (later IFN-γ), which was considered to be more closely associated with specific immune reaction. The importance of IFN-γ for the development of cellular immune responses is now firmly established. In contrast, the role of type I IFN in the modulation of T cell immunity has been only partially clarified. IFN-α, like IL-12 and IFN-γ, appears to favor the proliferation of Th1 lymphocytes (2). Several studies have emphasized the importance of IFN-α for the generation of CTLs. Mice lacking the type I IFN receptor by targeted deletion are unable to mount a significant CTL response to lymphocytic choriomeningitis virus (3). Importantly, exposure to type I IFNs during a primary immune response promotes both the clonal expansion and survival of CD8+ T cells responding to specific Ag in vivo (4). In addition to the well-known direct antiproliferative effects on tumor cells, type I IFNs have also been found essential for the generation of effective CTL responses against tumors. Neutralization of type I IFNs following injection of potent neutralizing Abs impaired the generation of a tumor-specific CTL response in murine models (5). These findings have been supported by murine studies involving tumor cells transduced to express IFN-α (6). Inoculation into mice with IFN-α-expressing tumor cells resulted in tumor rejection which was mediated by CD8+ T cells (6) and associated with the generation of long-lasting, tumor-specific memory. Type I IFN also inhibit the expression of the IL-12 p40 chain in dendritic cells, thereby limiting its production. IL-12, along with IL-18, is thought to be required for development of IFN-γ-producing CD4+ T cells (7), and thus at level IFN-α feedback, it may inhibit APCs. Further investigations are necessary to clarify the effects of IFN-α on immune responses.

In a previous study, we reported that IFN-α transduction of a poorly immunogenic tumor cell reduces tumorigenicity and leads to long-lasting tumor immunity (8). The studies reported here were performed to understand in greater detail the effect of IFN-α gene transduction on the induction of tumor-specific cellular immune responses. We show that IFN-α expressed by tumor cells enhances the generation of tumor-specific CTLs in vitro. Furthermore, we observed that IFN-α-transduced tumor cells promote the survival of a tumor-specific CTL line by preventing apoptosis. Both enhanced generation and survival of specific CTLs were found at comparable levels using tumor cells expressing the costimulatory molecule CD80.

Materials and Methods

Mouse

Female C57BL/6 mice, 6–8 wk old, were purchased from Taconic Farms (Germantown, NY) and were used in experiments when they were 8–12 wk old. Animals were maintained in a specific pathogen-free facility (Central Animal Facility, University of Pittsburgh, Pittsburgh, PA).

Cell lines, culture medium, and reagents

The murine colorectal adenocarcinoma (MC38) (9), the methylcholanthrene-induced fibrosarcoma (MCA205) (both of C57BL6 mouse origin), yeast artificial chromosome-1 (YAC-1) (10) lymphoma cells, and p815 thymoma cells were obtained from Dr. S. A. Rosenberg (Surgery Branch, 3 Abbreviations used in this paper: YAC-1, yeast artificial chromosome-1; MC38-CD80, MC38 cells transduced with CD80 gene; MC38-IFNα, MC38 cells transduced by guest on October 22, 2017 http://www.jimmunol.org/ Downloaded from
National Cancer Institute, Bethesda, MD). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 1 mM MEM sodium pyruvate, and 0.1 mM MEM nonessential amino acids (complete medium) in a humidified incubator with 5% CO₂ at 37°C. Cells were determined to be free of mycoplasma contamination (detected by Mycoplasma T. C., Gene-Probe, San Diego, CA). All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD).

A sheep polyclonal anti-mouse IFN-α-neutralizing Ab was a kind gift of Dr. G. Greber (Institut Gustave Roussy, Paris, France). 10 (11). Sheep IgG and rat IgG (Sigma, St. Louis, MO) were used as a control for sheep polyclonal anti-mouse IFN-α-neutralizing Ab and rat anti-mouse CD8α Ab, respectively. Flow cytometry was performed using FACSscan (Becton Dickinson, San Jose, CA). Anti-CD8α mAb was prepared from supernatant of the hybridoma TIB-105 (American Type Culture Collection, Manassas, VA) and was used for cytolytic assays and mixed lymphocyte/tumor proliferation assays to block interaction between TCR on CTLs and MHC class I molecules on tumor cells.

Retroviral construct and genetically modified tumor cell lines

A producer cell line for the retroviral vector SAM-mB7.1-EN was kindly provided by Dr. P. Hwu (National Cancer Institute, Bethesda, MD) (12). The construction and characterization of the DFG-mIFN-α2 retroviral vector has been described previously (13). Retroviral transfection was performed using standard approaches followed by selection of neomycin-resistant cells using the neomycin analogue, G418 (obtained from Life Technologies, Gaithersburg, MD), was done (14). The expression of mouse CD80 on the surface of transduced tumor cells (MC38-CD80) was confirmed by flow cytometry (FACSscan, Becton Dickinson) using a mAb specific for mouse CD80 (Becton Dickinson). The expression of mouse IFN-α2 was confirmed by ELISA (13). IFN-α2-transduced MC38 cells (MC38-IFNαNs) produced ~80 ng (2400 U)/10⁶ cells/48 h (8). Neomycin-resistant, gene-transduced MC38 (MC38-Neo) cells used for control cell line were obtained from Dr. Takashi Iwazawa (University of Pittsburgh, Pittsburgh, PA). Wild type (WT) MC38 (MC38-WT) cells or MC38-Neo did not express CD80 or IFN-α2. In vitro growth of these transduced cells was almost same as that of WT cells (8). No difference between survival of WT and that of genetically modified MC38 had been observed after gamma-irradiation.

Generation and maintenance of tumor-specific CTLs

Hyperimmunized mice were generated by initial injection with 1 x 10⁹ nonirradiated MC38-IFNαNs on day 0. Subsequently, tumor-free animals rejected a challenge with 3 x 10⁶ MC38-WT on day 35 and also 1 x 10⁶ MC38-WT on day 70. Splenocytes (1.5 x 10⁶ cells/ml) from these hyperimmunized mice were harvested on day 77 and were stimulated with WT or genetically modified irradiated (10,000 rad) MC38 tumor cells (1.5 x 10⁶ cells/ml). Seven days later, responder cells (5 x 10⁶ cells/ml) were restimulated with the same MC38 cell line (5 x 10⁶ cells/ml) for 4 days in medium containing 5 % IL-2 before the proliferation assay was performed. CTLs (5 x 10⁴ or 1 x 10⁵ cells/well) were co-cultured with 5 x 10⁶ or 1 x 10⁶ cells/well of WT or genetically modified gamma-irradiated (20,000 rad) MC38 cells or irrelevant MCA205 tumor cell line in 200 μl of complete medium in 96-well round-bottom plates for 3 days. The effect of IFN-α, 2000 U/ml of murine IFN-α2 or 15 μg/ml of anti-IFN-α Ab, which is capable of neutralizing over 5 x 10⁴ U/ml of IFN-α or, as a control, sheep IgG was added to some wells. Anti-CD8α mAb (5 μg/ml) was also added to block TCR-MHC interaction. Then, 1 μCi/well of [³²P]Thymidine was added to each well for 16 h. After an 18-h incubation, all cells were harvested onto glass fiber filter mats and [³²P]Thymidine uptake was assessed by liquid scintillation counting. Experiments were performed three or four times in triplicate.

DNA fragmentation (JAM) assays

MC38-specific CTLs (target cells) were labeled with [³H]Thymidine as previously described (15) and cocultured with or without tumor cells at indicated tumor to CTL ratios. Exogenous murine IFN-α2 (2000 U/ml) and anti-IFN-α-neutralizing Ab (15 μg/ml) were used to evaluate the effects of IFN-α. After 8 h, the cells were harvested onto glass fiber filter mats, and nuclear DNA-bound radioactivity was determined by liquid scintillation counting. Nuclear DNA-bound radioactivity of the 5 x 10⁶ CTLs incubated for 8 h without tumor cell was usually between 10,000 and 15,000 cpm. The nuclear contents of radiolabeled DNA are decreased as a result of DNA fragmentation associated with apoptotic cell death. The percentage of DNA fragmentation was calculated using the following formula (15):

\[
\text{DNA fragmentation} = \frac{\text{DNA-bound activity without tumor}}{\text{DNA-bound activity cocinbuted with tumor}} \times 100
\]

Statistical analyses

Significance was assessed by Student’s t test. The difference between groups was considered statistically significant when the p value was <0.05.

Results

IFN-α- and CD80-transduced MC38 cells generate MC38-specific CTLs in vitro from IFN-α-transduced MC38-immunized mice

Mice hyperimmunized to MC38 as a result of inoculation of MC38-IFNαNs and two subsequent rechallenges with a 3- to 10-fold greater number of MC38-WT were sacrificed, and splenocytes were harvested. Restimulation was performed twice in vitro with MC38-WT, -Neo, -CD80, or -IFNα and syngeneic splenocytes to assess the induction of tumor-specific cytotoxicity as described above in Materials and Methods. Cytotoxicity was determined in [⁵¹Cr]-release assays using MC38 and the lymphokine-activated killer-sensitive YAC-1 as targets. When MC38-WT or -Neo cells were used as stimulator cells, comparable cytolytic activity was noted for both MC38 and YAC-1 cells (Fig. 1, A and B). However, specific cytolyis for MC38 cells was clearly detected when MC38-CD80 (49.80% vs MC38 and 18.18% vs YAC-1; E:T = 40) or MC38-IFNα (50.01% vs MC38 and 15.87% vs YAC-1; E:T = 40) were used as stimulator cells (Fig. 1, C and D). IFN-α and CD80 transduction appears to enhance the generation of specific CTLs in vitro.

This CTL generation study using IFN-α-transduced tumor cells was extended to the murine methylcholanthrene-induced fibrosarcoma cell line MCA205. Restimulation of splenocytes harvested from MCA205-immune mice with MCA205 transduced with IFN-α or CD80 also led to tumor-specific cytolytic effectors (data not shown). In this model, we detected more nonspecific cytolytic activity due to lymphokine-activated killer activity against YAC-1 cells.
Characterization and specificity of MC38-specific CTLs

To further investigate the mechanism by which IFN-α expression of MC38 cells leads to the generation of tumor-specific CTLs, we generated an MC38-specific CTL line. Splenocytes harvested from hyperimmune mice were maintained by weekly stimulation with MC38-CD80, syngeneic splenocytes, and IL-2. After a third stimulation, cytotoxicity assays were performed against MC38-WT, YAC-1, and the irrelevant fibrosarcoma MCA205 cells. These effector cells revealed high specific cytolytic activity for MC38, but not for MCA205 and YAC-1. Importantly, substantial lysis of syngeneic naive splenocytes (5 × 10^5 cells/ml) and with irradiated (3000 rad) syngeneic naive splenocytes (5 × 10^5 cells/ml) and 50 IU/ml of recombinant IL-2. Seven days after the second stimulation, CTL assay was performed against MC38 cells (•) and YAC-1 cells (□) at indicated E:T ratios. Results are reported as mean percentage cytotoxicity ± SD.

IFN-α- and CD80-transduced MC38 cells enhance the proliferation of specific CTLs

After establishment of the MC38-specific CTL line MK-1 and the CTL clone cB4, we investigated the response to restimulation with WT or genetically modified MC38 tumor cells as well as with the irrelevant MCA205 tumor. We stimulated CTLs at a CTL-to-tumor ratio of 10 because CTLs proliferate more efficiently in vitro at this ratio. Proliferation of MK-1 CTLs was assessed by incorporation of [3H]Tdr during the final 18 h at the end of day 4. The uptake of [3H]Tdr by MK-1 cells was significantly enhanced in cultures set up with MC38-IFNα or MC38-CD80 compared with cultures set up with MC38-WT (p = 0.00215, MC38-IFNα; or p < 0.0001, MC38-CD80), MC38-Neo (p < 0.0001, MC38-IFNα and MC38-CD80), or MCA205 (Fig. 3A). Addition of exogenous 2000 U/ml IFN-α resulted in an enhanced proliferative response of MC38-specific CTL clone cB4 (p = 0.0040; Fig. 3B). When 15 μg/ml anti-IFN-α-neutralizing Ab, which is capable of neutralizing over 5 × 10^3 U/ml of IFN-α, was added in the assay, [3H]Tdr uptake by cB4 was decreased to almost the same level as the uptake when CTLs were incubated with MC38-WT (p = 0.0088). These results suggest that IFN-α- as well as CD80-transduced MC38 cells enhanced specific CTL proliferation in vivo. Because anti-CD8 mAb abrogated the proliferative response completely (p < 0.0004), TCR-MHC interactions seem to play a role (Fig. 3C).
CD80 signaling can indeed rescue CTLs from apoptotic cell death following Ag-specific stimulation. The mechanism by which IFN-α enhances CTL proliferation is not clear. Therefore, we performed JAM assays to determine whether IFN-α would also protect tumor-specific CTLs from apoptotic cell death. DNA fragmentation was detected when MK-1 cells were coincubated with MC38-WT and 2000 U/ml of mIFN-α2, MC38-IFNα and 15 μg/ml anti-IFN-α Ab which is capable of neutralizing over 5 x 10⁵ U/ml of IFN-α, or, as a control, sheep IgG. MK-1 cells were also coincubated with MC38 cells and 5 μg/ml anti-CD8 mAb. After 18 h of incubation with 1 μCi/well of [³H]Tdr, all cells were harvested onto glass fiber filter mats, and [³H]Tdr uptake was assessed by liquid scintillation counting. Experiments were done four times in triplicates with similar results. Results are reported as mean cpm ± SD.

FIGURE 4. CD80 and IFN-α expression decreases the apoptosis of specific CTLs following coincubation with relevant tumor targets. CTLs were labeled with [³H]Tdr by overnight incubation. Labeled MC38-specific CTLs (5-10 x 10⁵ target cells) were cocultured with or without tumor cells at indicated tumor-to-CTL ratios. A, CTLs were incubated with p815, YAC-1, MCA205, or MC38 cells at a tumor-to-CTL ratio of 80. B, Otherwise, CTLs were coincubated with WT or genetically modified MC38 cells. C, To evaluate the effects of IFN-α, CTLs were incubated with MC38-WT and exogenous murine IFN-α (2000 U/ml), MC38-IFNα and 15 μg/ml anti-IFN-α-neutralizing Ab, or sheep IgG. After 8 h, the cells were harvested onto glass fiber filter mats, and nuclear DNA-bound radioactivity was determined by liquid scintillation counting in proliferation assays. The percentage of DNA fragmentation as measured for apoptosis was calculated by following formula: percentage DNA fragmentation = [(DNA-bound activity without tumor) - (DNA-bound activity coincubated with tumor)] x 100 ÷ (DNA-bound activity without tumor). Experiments were performed three times with similar results. Results are reported as mean percentage DNA fragmentation ± SD.
were incubated with MC38-Neo and exogenous murine IFN-α2 (2000 U/ml). DNA fragmentation of CTLs was significantly less than that of CTLs incubated with MC38-Neo (p = 0.0111; Fig. 4C). DNA fragmentation of CTLs incubated with MC38-IFNα was significantly increased when anti-IFN-α-neutralizing Ab (15 μg/ml) was added in the assay (p = 0.0116; Fig. 4C). Exogenous IFN-α has the same antipapoptotic effect as shown for the transfectant in Fig. 4B.

Discussion

Transduction of IFN-α into poorly immunogenic murine tumor cell lines elicits a tumor-specific immune response in syngeneic immunocompetent hosts (6). Tumor rejection in vivo appears to depend upon a CD8+ cell-mediated immune response (6). We studied the mechanisms by which IFN-α gene expression promotes tumor-specific cellular immune responses. The effect of IFN-α-expressing MC38 tumor cells on tumor-specific CTLs was studied in vitro using splenocytes harvested from MC38-immune mice as responders. IFN-α mediates up-regulation of MHC class I molecules on tumor cells as well as enhanced killing activity of cytotoxic effector cells (16). Belardelli (2) also reported that IFN-α, like IL-12, promotes T cell differentiation toward a Th1 phenotype. Most importantly, type I IFNs can directly enhance T cell proliferation and expression of a memory T cell phenotype in vivo (4). Furthermore, Marrack et al. (17) demonstrated that type I IFNs diminish activated T cell apoptosis. Our results confirm and extend these observations. We found that IFN-α-expressing MC38 tumor cells were able to efficiently restimulate tumor-specific CTLs from splenocytes of MC38-immune mice in vitro, while MC38-WT or MC38-Neo cells were not. Effective restimulation of CTLs was observed at comparable levels when MC38-CD80 tumor cells were used. Furthermore, stimulation with MC38-IFNα tumor cells as well as MC38-CD80 tumor cells supported the proliferation of MC38-specific CTLs in vitro. Stimulation with MC38-WT or MC38-Neo MC38 was significantly less effective. The CD80/CD28 interaction is critical for survival, expansion, and delivery of signals allowing Ag-specific T cell responses and effector function (18–20). Cocultivation of CTLs with CD80-transduced tumor cells augmented the specificity of tumor-reactive CTLs in long-term culture (21). Proliferation of resting T cells in response to parasite-infected cells was also found to be dependent on expression of CD80 (22). Costimulatory signals delivered subsequent to CD80/CD28 interactions prevents T cell apoptosis during target cell lysis (23). Furthermore, ligation of CD28 by agonistic Abs enhances the expression of Bcl-XL and prevents activation-induced cell death (AICD) by apoptosis during activation of resting T cells (24).

Premature apoptosis of effector cells is proposed to be one of the major mechanism by which tumors escape host immune reaction (25). We hypothesized that IFN-α-expressing tumor cells as well as CD80-expressing tumor cells were capable of inhibiting AICD, which occurs after coinoculation of MC38-specific CTLs with MC38-WT tumor cells. This hypothesis was supported by DNA fragmentation assays, which demonstrated that IFN-α-transduced tumor cells, like CD80-transduced tumor cells, are able to prevent apoptotic cell death of tumor-specific CTLs. Apoptosis of MC38-specific CTLs after coinoculation with MC38 tumor cells in these assays was very likely due to AICD because only minimal DNA fragmentation was detected when CTLs were incubated with the irrelevant MCA205, YAC-1, or p815 tumor cells. Taken together, our results support the notion that IFN-α, like CD80, directly provides survival signals to tumor-specific CTLs.

We detected the DNA fragmentation of CTL at a tumor-to-CTL ratio of 80:1 but not 40:1. We believe that this reflects the physiological situation in vivo where a few CTLs attack a comparatively large amount of tumor cells. Our CTLs display a high specific cytolytic activity for MC38. Substantial lysis of MC38 was noted even at low E:T ratios. Because we performed 8-h JAM assays, MC38 cells were most likely killed by CTLs during the incubation. We presume that apoptosis of CTLs can be detected when CTLs are stimulated strongly by MC38 cells, i.e., when many tumor cells surround target CTLs. Therefore, a large number of MC38 cells may be required for CTL apoptosis to occur.

Because the natural IFN-α-producing cells have been recently identified as type 2 dendritic cell precursors (26), these cells provide stimuli of both IFN-α and CD80. Together with this identification, our findings suggest a novel role for these cells during the effector phase of the immune response.

Cytokine gene-engineered tumor vaccines allow paracrine delivery of cytokines into the tumor microenvironment, where they turn elicit antitumor effector cells (27, 28). We believe that this is the first evidence that IFN-α transduction of tumor cells enhances proliferation of tumor-specific CTLs and prevents AICD of the CTLs. Our results provide a scientific basis for the design of clinical trials involving IFN-α gene therapy for the treatment of cancer. IFN-α transduction with tumor cells should be considered for application in cancer clinical trials.

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