Immunomodulatory Gene Therapy With Interleukin 12 and 4-1BB Ligand: Long-Term Remission of Liver Metastases in a Mouse Model

Olivier Martinet, Vanadyia Ermekova, Jian Q. Qiao, Bernhard Sauter, John Mandeli, Lieping Chen, Shu-Hsia Chen

Background: The success of immunomodulatory cancer therapy is frequently hampered by the transient nature of the antitumor immune response. We have shown previously in a mouse model that interleukin 12 (IL-12) generates a strong natural killer (NK) cell-mediated antitumor response and reduces liver metastases induced by a colon carcinoma cell line. However, only a small percentage of the treated animals developed the cytotoxic T-lymphocytic response required for a long-term systemic antitumor immunity. 4-1BB is a co-stimulatory molecule expressed on the surface of activated T cells. Interaction of 4-1BB with its natural ligand (4-1BBL) has been shown to amplify T-cell (especially CD8+)-mediated immunity. In this study, we investigated the effects of adenovirus-mediated gene therapy delivering both IL-12 and 4-1BBL genes on mice with hepatic metastases induced by colon cancer cells. Methods: Syngeneic BALB/c mice received intrahepatic injection of poorly immunogenic MCA26 colon cancer cells. Various combinations of replication-defective adenoviruses expressing IL-12 and 4-1BBL genes were injected into the established liver tumors. Changes in tumor size and animal survival were then monitored. All statistical tests were two-sided. Results: The long-term survival rate of mice treated with the combination of IL-12 and 4-1BBL was significantly improved over that of animals in the control group (P = .0001). In vivo depletion of NK cells or CD8+ T cells completely abolished the long-term survival advantage of the IL-12 plus 4-1BBL-treated animals (P < .002). Moreover, the systemic immunity induced by this combination treatment protected these animals against a subcutaneous challenge with parental MCA26 cells. Conclusion: Adenovirus-mediated transfer of IL-12 and 4-1BBL genes directly into liver tumors resulted in tumor regression that required both NK and CD8+ T cells and generated a potent, long-lasting antitumor immunity. [J Natl Cancer Inst 2000;92:931–6]
have shown that in vivo adenovirus-mediated IL-12 (ADV/IL-12) gene transfer to established hepatic metastases induced a substantial reduction in tumor volume and a prolonged survival of treated animals compared with that of control animals. A small fraction of IL-12-treated animals was cured of their tumor and achieved long-term survival after tumor cell inoculation. NK cells were the early and major effector cells responsible for the survival benefit of IL-12-treated animals (18). Substantial initial tumor reduction, but no long-term survival, was achieved with IL-12 treatment in a study performed in tumor-bearing T-cell-depleted syngeneic animals and T-cell-deficient nude mice. These studies demonstrated that NK cells alone are essential for early immune rejection of tumor cells, but participation of T cells is required for long-term antitumor systemic immunity (18).

4-1BB ligand (4-1BBL) is a type II surface glycoprotein belonging to the tumor necrosis factor superfamily (19,20). Expression of 4-1BBL is restricted to the antigen-presenting cells, such as dendritic cells, macrophages, and activated B cells (21,22). This ligand interacts with its receptor (4-1BB) expressed on the surface of primed CD4+ and CD8+ T cells (23,24) in addition to T-cell receptor engagement and induces expansion, especially of CD8+ T cells (25). Enhanced 4-1BB/4-1BBL interaction has been shown to amplify T-cell-mediated immunity (26). Furthermore, systemic administration of agonistic anti-4-1BB antibodies can eradicate established subcutaneous tumors in mice (27) through activation of both CD4+ and CD8+ T cells.

In this study, we took a gene therapy approach to the problem and tested the antitumor consequences of intratumoral delivery of IL-12 and 4-1BBL genes. We used a syngeneic mouse model of liver metastases induced by a colon cancer cell line. We then analyzed the immune mechanisms underlying this combination treatment.

**METHODS**

**Cell cultures.** MCA26 cells (28), a chemically induced colon carcinoma line of low immunogenicity derived from a BALB/c background, were grown and maintained in high glucose minimum essential medium/Ham’s Medium F-12 (Sigma Chemical Co., St. Louis, MO). The 293 cells (adenoviral E1-transformed human embryonic kidney cells; American Type Culture Collection [ATCC]), Manassas, VA) were maintained in Dulbecco’s modified Eagle medium (Sigma Chemical Co.) (29). Culture media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Chemical Co.).

**Recombinant adenoviral vectors.** Construction of a replication-defective adenoviral vector bearing the murine IL-12 under the transcriptional control of the Rous sarcoma virus–long terminal repeat (RSV–LTR) promoter has been previously reported (17). The full-length mouse 4-1BB complementary DNA (cDNA) was obtained from plasmid pLSXHDm4-1BBL (26) by polymerase chain reaction (PCR) amplification by the use of appropriate primers with EcoRV and Nof links. The cDNA clone with the correct sequence was subcloned into the adenoviral shuttle vector (pAdL1/RSV–bpA) downstream of the RSV–LTR promoter at the Nof and EcoRV sites. Recombinant adenovirus was generated by co-transfection of the shuttle vector with pJM17, an E1-deleted adenovirus type 5 backbone vector, into 293 cells. The viral plaques were screened by flow cytometry analysis by use of a 4-1BB-human immunoglobulin (Ig) fusion protein (from L. Chen) and a secondary fluorescein isothiocyanate-labeled anti-human-Ig antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) on ADV/4-1BBL-transduced P815 plasmacytoma cells (ATCC). The viral titer (plaque-forming units [pfu]/mL) was determined by plaque assay in 293 cells (ATCC) (30). Expression of 4-1BBL was measured in vitro on ADV/4-1BBL-transduced JC breast cancer cells (ATCC) and CC36 (28) colon cancer cells, respectively.

**Mouse model with liver metastases.** All animal experiments were performed in accordance with the animal guidelines at Mount Sinai School of Medicine, New York, NY. Adult inbred female BALB/c mice (8–10 weeks old and weighing 18–20 g) (Taconic Farms, Germantown, NY) were anesthetized with an intraperitoneal injection of tribromoethanol (Avertin®); Aldrich Chemical Company, Milwaukee, WI). A midline laparotomy of 1 cm in length was performed, and the liver was exteriorized. MCA26 (7 × 10⁴) cells suspended in 10 μL Hanks’ balanced salt solution (Sigma Chemical Co.) were injected into the left lateral lobe. Seven days after the injection, liver tumors measuring between 4 × 4 and 6 × 6 mm in diameter were directly injected with ADV/IL-12, ADV/4-1BBL, and a control vector ADV/DVL312 in various combination and titers: 1) ADV/IL-12 at 1 × 10⁶ pfu plus ADV/4-1BBL 1 × 10⁶ pfu (n = 13); 2) ADV/IL-12 at 1 × 10⁶ pfu plus ADV/DVL312 at 1 × 10⁶ pfu (n = 3); 3) ADV/4-1BBL at 1 × 10⁶ pfu plus ADV/DVL312 at 1 × 10⁶ pfu (n = 11); and 4) ADV/DVL312 at 1 × 10⁶ pfu (n = 10). The recombinant adenoviruses were injected in 50–μL volumes of 10 mM Tris–HCl (pH 7.4) containing 1 mM MgCl₂/10% (vol/vol) glycerol and Polybrene (20 μg/mL) (all from Sigma Chemical Co.). Long-term survival and tumor response studies were performed to assess treatment outcome. For tumor response studies, the animals were killed 14 days after virus injection, and tumor diameters were measured. Tumor volume was calculated with the use of the following formula: a × b/2, where a = the largest diameter and b = the smallest diameter (31).

**Study of the immune cell subsets involved in the gene therapy with ADV/IL-12 and ADV/4-1BBL.** A long-term survival study of tumor-bearing animals treated with the combination ADV/IL-12 (1 × 10⁶ pfu) and ADV/4-1BBL (1 × 10⁶ pfu) was performed, with or without specific depletion of NK cells (with anti-asialo GM1 antibody treatment; Wako Pure Chemical Industries, Ltd., Richmond, VA) (32), depletion of CD4+ T cells (with GK 1.5 antibody treatment; ATCC) (33), or depletion of CD8+ T cells (with 2.43 antibody; ATCC) (34). A combination of rabbit IgG (Accurate Chemical and Scientific Corporation, Westbury, NY) and rat IgG (Caltag Laboratories, Burlingame, CA) was used in the control animals. The cell depletion was started the day before gene therapy delivery. Mice were given 50–100 μg of antibody intraperitoneally once per depletion per day. Antibodies for control or NK cell depletion were administered for 5 consecutive days and then every 5th day afterward for the duration of the experiment. Purified ascites fluid containing GK 1.5 or 2.43 antibodies was administered three times every other day, beginning 1 day before gene therapy delivery, and then every 5th day for the duration of the experiment. The efficacy of the immune subset depletion (>99%) with the use of these antibodies was confirmed by flow cytometry.

**Challenge experiments with parental tumor cells.** Long-term (>120 days) surviving animals treated by use of a gene therapy protocol with the combination IL-12 and 4-1BBL were given subcutaneous injections of 7 × 10⁴ MCA26 parental tumor cells in 100 μL Hanks’ balanced salt solution. Growth pattern and size of tumors were monitored. In vivo NK cell depletion (by administration of anti-asialo GM1 antibody) or total T-cell depletion (by administration of anti-Thy1.2 antibody, 53-6.72; ATCC) was performed in two different groups of long-term surviving mice. A combination of rabbit IgG and rat IgG was used in control long-term surviving animals. The schedule of antibody administration has been described in the prior experiment. “Naïve” BALB/c mice that have never been exposed to MCA26 cells were used to assess the growth of a subcutaneous MCA26 tumor.

**Statistical analysis.** The Wilcoxon rank-sum test was used to compare tumor responses in the different experimental groups. The survival of animals in long-term studies was estimated by the Kaplan–Meier method. Comparisons of survival curves of different groups were made by the log-rank test. To analyze the results of the kinetic study of subcutaneous tumor growth after challenge with parental tumor cells of mice with long-term survival after gene therapy, the Kaplan–Meier method was applied: For each animal, the average total tumor volume (ATTV) was calculated from day 6 to day 27. The ATTV equals the area under the curve (AUC) of the tumor volumes over time divided by the number of days (ATTV = AUC/number of days = AUC/27 – 6 = AUC/21). The ATTV represents the average height of the volume–time curve from day 6 to day 27, thereby giving an overall index of tumor size (34). Comparisons of ATTVs between two groups were performed by the Dunn multiple comparisons procedure for the Kruskal–Wallis test on the basis of four groups (35). All multiple comparisons for four groups in this article consisted of three comparisons (usually made up of a control group along with three treatment groups). Hence, to ensure an overall type I error rate of 0.05, each comparison was declared to be statistically significantly
different if the $P$ value was less than .05/3 = .017. All $P$ values were two sided.

**RESULTS**

**Assessment of ADV/4-1BBL Transgene Expression In Vitro and Tumor Response In Vivo**

*In vitro* 4-1BBL transgene expression was measured on JC breast cancer cells and CC36 colon cancer cells by use of flow cytometry analysis after a 48-hour co-incubation with ADV/4-1BBL or control vector ADV/DL312 at a multiplicity of infection of 200. The 4-1BBL molecule was expressed on 75.0% of JC cells (mean fluorescent intensity, 47.5) and on 76.8% of CC36 cells (mean fluorescent intensity, 56.3) of JC cells and 28.4% (mean fluorescent intensity, 23.9) and 76.8% (mean fluorescent intensity, 47.5) of CC36 cells were positive for 4-1BBL, respectively. *B* Antitumor effect of ADV/4-1BBL alone. MCA26 liver tumors measuring between 4 × 4 and 6 × 6 mm were injected with ADV/4-1BBL or ADV/DL312 (2 × 10^9 plaque-forming units/animal) (n = 17 mice/group). Animals were killed 14 days after virus injection, and the tumor size was measured. The median tumor volume was 252 (range, 32–1368) mm^3 and 968 (range, 108–2304) mm^3 for the 4-1BBL and DL312 groups, respectively ($P = .023$, Wilcoxon rank-sum test). Vertical bars represent 95% confidence intervals.

As shown in Fig. 2, all mice in the ADV/DL312 control group and the ADV/4-1BBL group died within 20–40 days after tumor implantation (compared with the group treated with the combination; $P < .0001$, log-rank test). Twenty-two percent (two of nine) of the animals treated with ADV/IL-12 alone compared with 62% (eight of 13) of the animals treated with ADV/IL-12 in combination with ADV/4-1BBL survived more than 120 days.

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 1. A) Adenovirus vector (ADV/4-1BB ligand (4-1BBL) assessment of transgene expression in vitro. The percentage of JC breast cancer or CC36 colon cancer cells positive for 4-1BBL 48 hours after transduction with ADV/4-1BBL or ADV/DL312 control vector (both at a multiplicity of infection of 200) was measured by flow cytometry analysis (1 × 10^6 cells counted) by use of a 4-1BB-human immunoglobulin (Ig) fusion protein and a secondary fluorescein isothiocyanate-labeled anti-human Ig antibody. When co-incubated with ADV/DL312 empty E1-deleted control vector or ADV/4-1BBL, 1.7% (mean fluorescent intensity, 15.7) and 17.5% (mean fluorescent intensity, 56.3) of JC cells and 28.4% (mean fluorescent intensity, 23.9) and 76.8% (mean fluorescent intensity, 47.5) of CC36 cells were positive for 4-1BBL, respectively. B) Antitumor effect of ADV/4-1BBL alone. MCA26 liver tumors measuring between 4 × 4 and 6 × 6 mm were injected with ADV/4-1BBL or ADV/DL312 (2 × 10^9 plaque-forming units/animal) (n = 17 mice/group). Animals were killed 14 days after virus injection, and the tumor size was measured. The median tumor volume was 252 (range, 32–1368) mm^3 and 968 (range, 108–2304) mm^3 for the 4-1BBL and DL312 groups, respectively ($P = .023$, Wilcoxon rank-sum test). Vertical bars represent 95% confidence intervals.

Fig. 2. Long-term Kaplan–Meier survival of BALB/c mice bearing syngeneic MCA26 liver metastases treated with the combination of adenoviral vector (ADV/interleukin 12 (IL-12) and ADV/4-1BB ligand (4-1BBL). Animals with tumors measuring between 4 × 4 and 6 × 6 mm were randomly assigned to one of four groups: group 1 (●) = ADV/IL-12 (1 × 10^8 plaque-forming units [pfu]) + ADV/4-1BBL (1 × 10^9 pfu) (n = 13); group 2 (■) = ADV/IL-12 (1 × 10^8 pfu) + ADV/DL312 (1 × 10^9 pfu) (n = 9); group 3 (▲) = ADV/4-1BBL (1 × 10^8 pfu) + ADV/DL312 (1 × 10^9 pfu) (n = 11); and group 4 (▲) = ADV/DL312 (1.1 × 10^9 pfu) (n = 10). The viral vectors were injected directly into the tumor. Eight (62%) of 13 animals in the group treated with the combination of IL-12/4-1BBL compared with two (22%) of nine animals in the IL-12 group were alive at 120 days after tumor inoculation ($P = .015$, log-rank test). All of the animals in the 4-1BBL- and DL312-alone groups died between 20 and 40 days (compared with the combination treatment group; $P = .0001$, log-rank test). Vertical bars represent 95% confidence intervals.
days after tumor inoculation \((P = .015\), log-rank test).

**Requirements of Both NK Cells and CD8+ T Cells for Long-Term Antitumor Immunity**

To study the effector cells that are responsible for the antitumor immunity generated by the combination ADV/IL-12 plus ADV/4-1BBL treatment, an in vivo depletion experiment was conducted (Fig. 3). All of the animals in the NK cell-depletion \((n = 10)\) and CD8 cell-depletion \((n = 10)\) groups died between 20 and 30 days after tumor implantation because of tumor progression at the liver and at distant sites. Mice in the CD4 cell-depletion \((n = 8)\) and the control Ig \((n = 8)\) groups showed no difference in long-term survival \((P = .6\), log-rank test). The differences observed between control Ig and NK and control Ig and CD8 cell-depletion groups are statistically significant \((P = .0003\) and .002, respectively; log-rank test). Those results indicate that both NK cells and CD8+ T cells are required for the long-term tumor remission induced by the IL-12/4-1BBL gene therapy.

**Persistence of Long-Term Systemic Antitumor Immunity**

To test the persistence of a systemic antitumor immunity, long-term \((>120\) days) surviving animals after gene therapy were challenged with a subcutaneous injection of parental MCA26 tumor cells. The tumor growth was observed for a 4-week period in four groups of animals: 1) naive mice \((n = 7)\), 2) NK cell-depleted mice \((n = 9)\), 3) T-cell-depleted mice \((n = 5)\), and 4) control Ig-treated mice \((n = 7)\). Mice from groups 2–4 were long-term surviving animals after gene therapy treatment with the combination IL-12 and 4-1BBL. One hundred percent of T-cell-depleted and naive mice formed a tumor. Compared with the control Ig group \((P = .015\) and .005, respectively; Fisher’s exact test). In the NK cell-depleted group, 77.7\% (seven of nine) of the animals grew tumors, while only 14.2\% (one of seven) mice in the control Ig-treated group did so (Fig. 4, A) \((P = .041\), not significant tumor progression; Fisher’s exact test). The mean tumor volumes calculated as ATTV for different groups at different time points are shown in Fig. 4, B. The differences in ATTV observed between the control Ig-treated group and the T-cell-depleted and naive groups are statistically significant \((P < .002\) level, Dunn multiple comparisons procedure for the Kruskal–Wallis test). The difference between control Ig-treated and NK cell-depleted groups is not statistically significant after adjustment for multiple comparisons. To test systemic antigenic specificity by use of the same experimental design, naive mice and long-term surviving mice after ADV/IL-12/ADV/4-1BBL combination treatment were challenged with a subcutaneous injection of unrelated JC breast cancer cells. One hundred percent of naive mice \((n = 7)\) and 75\% of long-term surviving mice \((n = 8)\) formed a JC tumor \((P = .47\), Fisher’s exact test). These results confirm the presence of a specific antitumor immune response in the combination-treated animals.

**DISCUSSION**

In a previous study (17), we have shown the efficacy of intratumoral administration of ADV/IL-12 monotherapy in a colon cancer liver metastases model with respect to tumor regression and survival advantages. Nevertheless, 75\% of ADV/IL-12-treated animals died secondary to tumor progression. IL-12 was found to induce a strong tumor-specific NK cell response leading to initial tumor regression (18). T-cell activation, however, being essential for long-term antitumor immunity, in addition to the observed NK cell stimulation, was found only in 25\% of the ADV/IL-12-treated animals, which showed long-term survival \((>120\) days after tumor inoculation) (18). Conversely, animals that did not have T-cell activation died within 50 days after tumor implantation.

Therefore, in this study, we tested the potential of an adenoviral-mediated intratumoral gene transfer of the combination IL-12 and 4-1BBL, a powerful T-cell co-stimulatory molecule, in an orthotopic tumor model of metastatic colon carcinoma. 4-1BBL alone may have induced a T-cell-mediated antitumor immune response that led to a statistically significant delay in tumor growth. However, this T-cell-mediated antitumor response did not persist, and all of the animals treated with 4-1BBL alone eventually died 30–40 days after tumor inoculation. In combination with IL-12, 4-1BBL may have induced a stronger stimulation and proliferation of T cells. The combination treatment led to a statistically significant higher percentage of long-term surviving animals and the development of a potent antitumor sys-

![Fig. 3. Study of immune mechanisms following the administration of the combination of adenoviral vector (ADV)/4-1BB ligand (4-1BBL) and ADV/interleukin 12 (IL-12). An in vivo depletion of various immune effector cells was performed on tumor-bearing animals treated with the combination ADV/IL-12 and ADV/4-1BBL. The long-term survival was compared between four different groups: group 1 (■) = natural killer (NK) cell depletion \((n = 10)\); group 2 (▲) = CD4+ T-cell depletion \((n = 8)\); group 3 (▲) = CD8+ T-cell depletion \((n = 10)\); and group 4 (●) = control immunoglobulin (Ig) \((n = 8)\). All of the animals in the NK cell- and CD8+ T-cell-depleted groups died between 20 and 30 days after tumor implantation. CD4+ T-cell-depleted and control Ig-treated animals showed comparable long-term survival without statistically significant difference \((P = .6\), log-rank test). The differences between the control Ig group and the NK- or CD8-depleted groups, however, are statistically significant \((P = .0003\) and .002 respectively; log-rank test). Vertical bars represent 95\% confidence intervals.](image-url)
tumor immunity induced by the combi-
role of CD8+ T cells in the long-term an-
group. These results stressed the critical
dependence on CD8+ T cells for long-term antitumor immunity. Compared with T-cell-depleted mice because of their lack of CD8+ T cells, which are essential for the initial antitumor effect mediated by NK cells, CD8+ T cells played the central role in long-term systemic antitumor immunity.

Depletion of either cell subset at the time of gene treatment led to rapid death of the NK cell- or T-cell-depleted animals because of tumor progression. In accordance, NK cell or T-cell depletion at the time of gene treatment led to rapid death of the NK cell- or T-cell-depleted animals because of tumor progression. In contrast, the combination IL-12/4-1BBL achieved long-term survival in 62% of the treated animals. IL-12 and 4-1BBL in combination act synergistically and had a better efficacy than either treatment alone. Moreover, as shown in the subcutaneous challenge of long-term surviving animals with parental and non-related tumor cells, the induced systemic antitumor immunity is persistent and specific. The mechanism of action of the combination IL-12/4-1BBL at the cellular level is not known. Schematically, IL-12 stimulates principally NK cells, while 4-1BBL induces proliferation of activated T cells. As shown previously, 4-1BB agonistic antibody or 4-1BBL was able to induce an immunomodulatory function of NK cells. The effect of 4-1BBL on IL-12-activated NK cells and vice versa is not known, although the main immune effector cells explaining the synergistic action of IL-12 and 4-1BBL could be the NK cells that could be triggered either by engagement of 4-1BB or by T cells. By secreting cytokines like IL-12, granulocyte–macrophage colony-stimulating factor, or IFN γ through cell-to-cell contact, NK cells could potentiate the induced immune response by stimulating antigen-presenting cells and T cells.

In conclusion, adenoviral-mediated intratumoral delivery of IL-12 and 4-1BBL genes in combination could represent a future treatment modality for metastatic colon carcinoma in humans.

REFERENCES


