IFN-γ-dependent type 1 immunity is crucial for immunosurveillance against squamous cell carcinoma in a novel mouse carcinogenesis model

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3-Methylcholanthrene (MCA)-induced sarcomas have been used as conventional tools for investigating immunosurveillance against tumor development. However, MCA-induced sarcoma is not always an ideal model for the study of the human cancer system because carcinomas and not sarcomas are the dominant types of human cancers. To resolve this problem, we established a novel and simple method to induce mouse squamous cell carcinomas (SCCs). As well known, the subcutaneous injection of MCA caused the formation of sarcomas at 100% incidence. However, we here first succeeded at inducing SCC at 60% of incidence within 2 months by a single intra-dermal injection of MCA. Using this primary SCC model, we demonstrated the critical role of interferon (IFN)-γ-dependent type 1 immunity in immunosurveillance against SCC from the following results: (i) The incidence of SCC was accelerated in IFN-γ-deficient mice compared with that in wild-type mice; (ii) In vivo injection of CpG-oligodeoxynucleotides (CpG-ODN) caused a marked reduction in the incidence of SCC in parallel with the activation of type 1-dependent antitumor immunity and (iii) The antitumor activity of CpG-ODN was significantly decreased in IFN-γ-deficient mice. Thus, our established MCA-induced mouse SCC model could be a powerful tool for evaluating immunosurveillance mechanisms during the development of SCC and might result in a novel strategy to address immunosurveillance mechanisms of human cancer.

Introduction

Since the cancer immunosurveillance hypothesis was proposed by Paul Ehrlich, Macfarlane Burnet and Lewis Thomas (1–3), tumor immunologists have asked whether the host immune system prevents tumor growth. Several groups have redressed this issue by providing strong evidence for the existence of an effective cancer immunosurveillance process in mice using mainly chemical carcinogenesis experiments (4–14). Cramer’s group established the chemical carcinogenesis method to induce skin carcinoma in mice ~50 years ago (15,16), but it took ~6 months of repeated skin application of 3-methylcholanthrene (MCA) to induce the tumors, which has made it difficult to apply this model to the evaluation of host immunosurveillance mechanisms during carcinogenesis. Thus, most investigators adopted improved methods to induce primary tumors by the subcutaneous (s.c.) injection of MCA. The method generates carcinoma in mice and has been used as a primary tumor model for evaluating the role of immunosurveillance during tumorigenesis (4–11). However, this primary tumor system is not an ideal one to address human cancer immunosurveillance because carcinoma is the dominant type of human cancer. Therefore, establishment of a simple method to induce mouse primary carcinoma is essential to address the potentially crucial role of immunosurveillance mechanisms against human cancer. The model is also expected to contribute to the development of a novel strategy for the immunotherapy of cancer.

Our working hypothesis was that injection of MCA into fibroblast-rich s.c. tissue causes fibrosarcoma, whereas injection of MCA into epithelial cell-rich intra-dermal (i.d.) tissue may result in the formation of carcinomas. Consistent with our hypothesis, it was initially demonstrated that a single i.d. injection of MCA caused the induction of mouse skin squamous cell carcinomas (SCCs). Using this novel model, we demonstrated that (i) CpG-oligodeoxynucleotides (CpG-ODN), the Toll-like receptor 9 ligand, prevent the development of MCA-induced primary mouse SCC; (ii) Interferon (IFN)-γ is involved in immunosurveillance against MCA-induced SCC and (iii) IFN-γ plays a critical role in the activation of CpG-ODN-induced antitumor immunity during the prevention of the development of primary SCC. Thus, our established MCA-induced primary carcinoma model can contribute to the investigation of immunosurveillance mechanisms during carcinogenesis, and result may be useful for developing a novel therapeutic strategy for human cancer.

Materials and methods

Mice

Wild-type (WT) BALB/c mice at 6–8 weeks of age were obtained from Charles River Japan (Yokohama, Japan). IFN-γ-deficient BALB/c mice were kindly provided by Dr. Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). All mice were maintained under specific pathogen-free conditions and used for the following experiments.

Carcinogenesis

WT BALB/c and IFN-γ-deficient BALB/c mice were injected s.c. or i.d. at the right flank with 0.1 ml of corn oil containing 500 μg of MCA (Sigma-Aldrich, Tokyo, Japan). The mice were monitored twice a week for the development of primary tumors. Tumors >100 mm³ that showed subsequent progressive growth were counted as positive. To examine the effect of CpG-ODN on carcinogenesis, 2 weeks after i.d. injection of MCA, the mice were injected with saline i.d. near the draining lymph node (DLN) or with 50 μg of CpG-ODN.

Histology and immunohistochemistry

Primary tumor tissues were removed from each MCA-injected mouse. MCA-induced inflammatory tissues were prepared for analysis 10 or 26 days after i.d. injection of MCA. Paraﬁn-embedded tissue sections (5 μm) were stained with hematoxylin and eosin. Tumor tissues were embedded in optimal cutting temperature compound and frozen in liquid nitrogen. Cryosections (5–7 μm) were fixed in cold acetone for 10 min and stained with purified anti-cytokeratin, anti-vimentin (Dako, Carpinteria, CA) or anti-CD8α (BD Biosciences, Franklin Lakes, NJ) monoclonal antibody (mAb) at room temperature for 1 h. The sections were then incubated with peroxidase-conjugated secondary antibody (Histone semiplastin MAX PO; Nichirei, Tokyo, Japan) at room temperature for 10 min. Peroxidase reactivity was detected with diaminobenzidine (Sigma-Aldrich, Tokyo, Japan). The serial section was stained with hematoxylin and eosin. The number of CD8⁺ cells was counted under light microscopy at x200 magnification. The means and standard deviations per 12 high power fields per group were calculated and are indicated in the figure.

Cytotoxic assay

Reverse antibody-dependent cell-mediated cytotoxicity of lymphocytes was measured by 4 h 51Cr-release assay as described previously (17). Briefly, lymphocytes were collected from the DLN of saline- or CpG-ODN-injected mice 26 days after MCA injection and incubated with 51Cr-labeled Fc-receptor-positive P815 cells in the presence or absence of soluble anti-CD3 mAb (145-2C11) (BD PharMingen, San Diego, CA) for 4 h. Percentage of cytotoxicity was calculated as described previously (18).

Abbreviations: CpG-ODN, CpG-oligodeoxynucleotides; DLN, draining lymph node; i.d., intra-dermal; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; MCA, 3-methylcholanthrene; s.c., subcutaneous; SCC, squamous cell carcinoma; WT, wild-type.

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Enzyme-linked immunosorbent assay
Lympocytes (5 × 10^5 cells) prepared from DLN were cultured in 250 ml of Roswell Park Memorial Institute medium (Sigma) containing 10% fetal calf serum (BD Biosciences) plus penicillin and streptomycin in 96-well flat-bottom plates. The cells were stimulated with soluble anti-CD3 mAb, anti-CD3 mAb plus interleukin (IL)-2 (kindly donated by Dr T. Sawada, Shionogi Pharmaceutical Institute Co. Ltd, Osaka, Japan) or IL-2 plus IL-12 (by Wyeth Research, Cambridge, MA) for 36 h. IFN-γ levels in the culture supernatants were determined by OptEIA mouse enzyme-linked immunosorbent assay system (BD PharMingen).

Flow cytometric analysis
For the detection of intracellular cytokines, the lymphocytes from DLN of saline- or CpG-ODN-injected mice 26 days after MCA injection were stimulated with anti-CD3ε mAb (145-2C11, BD Biosciences) in 96-well flat-bottom plates for 6 h and treated with Brefeldin-A for the last 2 h. The stimulated cells were first stained with phycoerythrin–Cy7–anti-CD4 mAb (MR4.5, eBioscience, San Diego, CA) and allophycocyanin–Cy7–anti-CD8a mAb (53-6.7, BD Biosciences) and then fixed with 4% paraformaldehyde phosphate buffer solution (Wako, Osaka, Japan). After treatment with permeabilizing solution [50 mmol/l NaCl, 5 mmol/l ethylenediaminetetraacetic acid, 0.02% NaN3, and...
0.5% Triton X-100 (pH 7.5)); the cells were stained with fluorescein isothiocyanate–anti-IFN-γ mAb (XMG1.2, BD Biosciences) and phycoerythrin–anti-IL-4 mAb (1B11, BD Biosciences). Fluorescence signals from the cells were acquired by FACSCanto (BD Biosciences) and analyzed with FACSDiva software and CellQuest software (BD Biosciences).

**Real-time polymerase chain reaction**

Total RNA was extracted from inflamed tissues of the MCA-injected mice at day 26 using the Isogen RNA extraction kit (Nippon Gene, Toyama, Japan) and RNeasy Mini kit (QIAGEN, Germantown, MD), according to the manufacturer’s instructions. Complementary DNA was prepared from the total RNA with reverse transcriptase (Invitrogen, Carlsbad, CA), oligodeoxynucleotide and deoxyxynucleoside triphosphate mixture (Promega, Madison, WI) after digesting genomic DNA using deoxyribonuclease I amplification grade (Invitrogen). The indicated complementary DNA was specifically amplified by thermal cycler (LightCycler, Roche, Indianapolis, IN) using the corresponding primer pairs and probes for mouse IFN-γ and β-actin. The sequences used were as follows: IFN-γ (sense) 5'-GGTGCTATCATGATGATTC-3', (anti-sense) 5'-GCTTCATTGAGGTGATGC-3', (probe) 5'-TTTGAGGTCAACAAACCCACAGGTCCA-3' and β-actin: (sense) 5'-AGCAGTCACTGACCACT-3', (anti-sense) 5'-TCTCCGGAGTCCATCACAATG-3', (probe) 5'-TGTCCCTGTATGCCTCTGGTCGTACCA-3'. Sample signals were normalized to the housekeeping gene β-actin according to the ΔΔCt method: ΔCt = ΔCt sample − ΔCtreference. Percentages against the WT control sample were then calculated for each sample.

**Statistical analysis**

Significant differences in the results were determined by the Mann–Whitney U-test or Student’s t-test. P < 0.05 was considered significant in the present experiments.

**Results**

**Induction of mouse primary skin SCC by i.d. injection of MCA**

It has been reported that s.c. injection of MCA caused the generation of sarcomas. However, here, we tried to induce MCA-induced SCC by i.d. injection of MCA, based on our working hypothesis that i.d. injection of MCA might induce chronic inflammation around the injection site and somatic mutagenesis of the surrounding dermal epithelial cells. WT BALB/c mice were injected s.c. or i.d. with MCA (500 µg) and monitored for the growth of tumors. Tumors were observed 10 weeks after s.c. injection of MCA, while this was greatly accelerated by i.d. injection of MCA and tumors appeared at 6 weeks (Figure 1A). Twelve weeks after MCA injection, 100% of mice developed primary tumors after i.d. injection, whereas s.c. injection induced tumors in ~25% of the MCA-treated mice. When tumor size reached 500 mm³, the tumor tissues were collected for histological analysis. As shown in Figure 1B, the tumor tissues from mice treated with s.c. injection of MCA expressed vimentin, a fibroblast marker, whereas they did not express cytokeratin, an epithelial cell marker. This histological analysis indicated that 100% of the tumors induced by s.c. injection of MCA were fibrosarcomas. Notably, however, ~60% of the tumors from mice injected i.d. with MCA expressed cytokeratin, but not vimentin. The tissues also showed infiltration of stroma and keratinization of epithelial cells at the MCA-injection sites. From these characteristic features, we concluded that i.d. injection of MCA successfully induced SCC and sarcomas at a ratio of 60–40%, respectively. However, s.c. injection of MCA induced only sarcomas (Figure 1C).

As clearly demonstrated in the result of the kinetics study on primary tumor development (Figure 1A), we found that the appearance of tumors in mice treated with s.c. injection of MCA was much earlier than that of tumors in mice treated with s.c. injection of MCA. It was also demonstrated that fibrosarcomas induced by i.d. injection of MCA grew more rapidly than those induced by s.c. injection of MCA (Figure 1D and E). However, SCC appeared more rapidly than fibrosarcoma in mice injected i.d. with MCA. Thus, our MCA-induced SCC model is a very rapid and simple carcinogenesis model, which will be useful for investigating immunosurveillance mechanisms during carcinogenesis.

**The critical role of IFN-γ for preventing the growth of primary mouse skin SCC**

The role of IFN-γ in immunosurveillance against skin SCC is still largely unknown. Therefore, to define a critical role for IFN-γ during MCA-induced SCC formation in our established model, MCA was i.d. injected into WT or IFN-γ-deficient BALB/c mice and then the incidence of SCC was monitored. As shown in Figure 2, there were no differences between WT and IFN-γ-deficient mice in the onset of SCC, and tumors appeared 7 weeks after MCA injection. In contrast, the incidence of SCC in IFN-γ-deficient mice significantly increased compared with that in WT mice. We also confirmed that the incidence of sarcomas was not altered (data not shown). Next, to investigate inflammatory events at the MCA-injected site, we performed hematoxylin and eosin staining. As a result, there were no significant differences in inflammatory cell recruitment and thickening of the epithelium between WT and IFN-γ-deficient mice, suggesting that the increase in tumor incidence in the IFN-γ-deficient mice was not derived from distinct inflammatory responses at the MCA-injected site (supplementary Figure 1 is available at Carcinogenesis Online). These evidences clearly indicated that IFN-γ plays a critical role in immunosurveillance against MCA-induced primary SCC.

**CpG-ODN administration significantly suppresses the incidence of MCA-induced SCC development**

We further investigated whether CpG-ODN, a strong activator of type 1 immunity (17,19–22), affected the incidence of MCA-induced SCC in our established model. WT BALB/c mice were i.d. injected with MCA and then treated with an i.d. injection of CpG-ODN every 10 days. The CpG-ODN administration significantly inhibited tumor development (Figure 3A-a). Interestingly, both the onset and the incidence of SCC development were markedly inhibited by the treatment with CpG-ODN (Figure 3A-b), though the development of fibrosarcomas was not significantly affected (Figure 3A-c). The final frequency of SCC development greatly decreased in CpG-ODN-treated mice (37.0%) compared with untreated control mice (58.3%) (Figure 3B). These results first demonstrated that CpG-ODN administration could suppress the development of primary SCC in addition to sarcoma.
The critical role of CpG-ODN-induced type 1 immunity for enhancing immunosurveillance against MCA-induced SCC

In order to evaluate the immunosurveillance mechanisms enhanced by CpG-ODN, we examined the effect on cell-mediated cytotoxicity and IFN-γ production of lymphocytes from MCA-injected mice. Ten weeks after the MCA injection, lymphocytes were collected from DLN of the MCA-injected site and stimulated with the indicated agents. As a result, IFN-γ production by lymphocytes from the CpG-ODN-treated group was significantly enhanced compared with those from the control group (Figure 4A). In addition, we found that CpG-ODN treatment significantly increased the percentages of IFN-γ-producing cells in the CD8⁺ T cell fraction, but not in the CD4⁺ T cell population (Figure 4B). Consistent with the CD8⁺ T cell activation, lymphocytes from the CpG-ODN-treated mice showed higher
reverse antibody-dependent cell-mediated cytotoxicity against P815 cells than those from control mice (Figure 4C). These results indicate that CpG-ODN treatment enhances IFN-γ-mediated type1 immunity in the DLN.

Next, we evaluated the activation of type 1 immunity in the MCA-injected local site. Gene expression analysis of the MCA-induced inflammatory tissues revealed that IFN-γ expression levels were markedly increased by CpG-ODN injection (Figure 5A). Furthermore, we found that inflammatory tissues prepared from CpG-ODN-treated mice exhibited increased number of infiltrating CD8+ T cell than those from control mice (Figure 5B).

IFN-γ production is required for the CpG-ODN-induced prevention of MCA-induced primary SCC

Finally, we investigated the mechanisms of CpG-ODN-induced potentiation of immunosurveillance against MCA-induced SCC. CpG-ODN treatment induced high levels of IFN-γ production by lymphocytes from the MCA-injected mice (Figures 4 and 5). Therefore, we addressed whether CpG-ODN-induced IFN-γ plays a crucial role in preventing the incidence of MCA-induced SCC. WT mice or IFN-γ-deficient mice were injected i.d. with MCA and then CpG-ODN was administrated as described in the Materials and Methods.
Tumor development in MCA-injected WT mice was greatly inhibited by the treatment with CpG-ODN (Figure 6A-a). However, the inhibitory effect of CpG-ODN on the development of SCC was not significant in the case of IFN-γ-deficient mice injected i.d. with MCA (Figure 6A-b). The inhibitory effect of CpG-ODN on tumor incidence was significantly reduced in IFN-γ-deficient mice compared with that in WT mice (Figure 6B). From the evidence, it was demonstrated that CpG-ODN treatment enhanced immunosurveillance against SCC in an IFN-γ-dependent manner.

Discussion

In the present paper, we report on the establishment of a novel, simple and rapid skin carcinogenesis model, inducing primary SCC by a single i.d. injection of MCA. In order to address the skin carcinogenesis mechanisms, many investigators have utilized dimethylbenz[a]anthracene and/or phorbol 12-myristate 13-acetate as carcinogens or promoters, respectively, to induce skin carcinomas, instead of MCA (5,12,13). However, these models are not ideal for evaluating immunosurveillance mechanisms during carcinogenesis because mice have to be repeatedly treated with carcinogens or promoters for long term to induce skin carcinomas. Thus, our novel method, which can induce SCC within 2 months by a single i.d. injection of MCA, is more useful.
than dimethylbenz(a)anthracene/phorbol 12-myristate 13-acetate carcinogenesis models to evaluate the immune responses during carcinogenesis (Figure 1). Interestingly, the MCA i.d. injection route induces more rapid appearance of tumors (a mixture of 60% carcinoma and 40% sarcoma) compared with the s.c. injection route, which is preferable to induce sarcomas (Figure 1A). One possible explanation for this phenomenon is that dermal epithelial cells might be more sensitive to MCA-induced mutagenesis than s.c. fibroblasts. However, this is unlikely because sarcoma cells induced by the i.d. injection route of MCA also exhibit a more rapid appearance than sarcoma cells induced by the s.c. injection route (Figure 1D and E). Therefore, i.d. injection of MCA appears to favor primary tumor induction possibly because it is (i) a preferable route for inducing a rapid MCA-induced SCC development, which may be because of chronic inflammation at the MCA-injected dermal site, and (ii) a preferable route for rapid induction of tumor, which may be because of long-term duration of MCA at the injection site.

Cancer immunosurveillance mechanisms during development of carcinomas in humans is largely not understood, since many investigators demonstrated the involvement of various cytokines and cell populations in immunosurveillance against sarcomas using the carcinogenesis model based on s.c. injection of MCA. In this article, we proposed that our novel MCA-induced SCC carcinogenesis model allowed us to evaluate immunosurveillance mechanisms against not only carcinoma but also sarcoma. We initially found that the incidence of carcinoma was increased in IFN-γ-deficient mice compared with WT mice after i.d. injection of MCA (Figure 2). These results are consistent with other spontaneous carcinogenesis models using IFN-γ or signal transducers and activator of transcription recombinase activating gene 2-deficient mice (6,23), indicating that IFN-γ-dependent immunosurveillance contributes to not only spontaneous development of carcinomas but also carcinogen-induced skin carcinomas. Moreover, as shown in Figure 2, IFN-γ-deficient mice exhibited an increased incidence of carcinoma but not delayed onset of cancer, suggesting that IFN-γ-dependent immune responses might be effective in the promotion phase rather than in the initiation phase during carcinogenesis.

CpG-ODN, a potent adjuvant for activating type 1 innate immunity, has been demonstrated to induce a strong antitumor immunity in various grafted tumor models (17,19–21). So far, it has been reported that CpG-ODN and other type 1 immunity-associated agents, such as IL-12 and γδT cells, inhibited primary tumor development (10,11,24). However, the detailed mechanisms underlying type 1 immunity-dependent immunosurveillance remained unknown. Here, we first demonstrate that CpG-ODN treatment inhibits the development of MCA-induced primary SCC with an increase in the expression level of IFN-γ and the number of infiltrating CD8+ T cells and that its inhibitory effect is significantly reduced in IFN-γ-deficient mice (Figures 3–5). Moreover, we revealed that CpG-ODN induced IFN-γ-producing CD8+ T cells but not CD4+ T cells in DLN (Figure 4B), consistent with our previous report that the induction of tumor-specific cytotoxic T lymphocyte by CpG-ODN vaccination was independent of CD4+ T cells. Thus, these results suggested that IFN-γ/CD8+ T cells, induced by CpG-ODN stimulation in the DLN, would play a role as effector cells in cancer immunosurveillance against primary MCA-induced SCC. However, it has been reported that CpG-ODN could eradicate transplanted tumors by the activation of natural killer cells, natural killer T cells and/or CD8+ T cells in immunotherapeutic models (20,21,25,26). Therefore, we need to evaluate the involvement of natural killer/natural killer T cells in the elimination of MCA-induced primary tumors after CpG-ODN treatment.

Interestingly, we found that CpG-ODN exhibits a superior tumor inhibitory activity against SCC rather than sarcoma (Figure 3A). Moreover, the development of carcinoma was promoted in IFN-γ-deficient mice but that of sarcoma was not (Figure 2 and data not shown). These findings strongly indicated that there might be differential immunosurveillance mechanisms via IFN-γ-induced type 1 immunity between sarcomas and carcinomas. In our novel primary carcinoma model, we revealed that CpG-ODN appears to exhibit its antitumor activity against MCA-induced SCC through the activation of type 1-dependent antitumor immunity including cell-mediated cytotoxicity and cytokine production (Figures 3–5). Using IFN-γ-deficient mice, it is also demonstrated that IFN-γ plays a pivotal role in the prevention of MCA-induced SCC by CpG-ODN (Figure 6). Together, we conclude that CpG-ODN inhibits the growth of primary MCA-induced SCC through the activation of IFN-γ-dependent type 1 immunity. Thus, we believe that our established novel, simple and rapid MCA-induced SCC model will contribute to further evaluation of immunosurveillance mechanisms against carcinomas and the development of a novel immunotherapy for human carcinomas.

Supplementary material
Supplementary figure 1 can be found at http://carcin.oxfordjournals.org/

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