Effective Response of the Peritoneum Microenvironment to Peritoneal and Systemic Metastasis from Colorectal Carcinoma

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Abstract

We here document discovery of a new and simple model of tumor seeding involving the mouse peritoneum. Irradiated tumor cells administered by i.p. injection provided effective vaccination against peritoneal carcinomatosis and distal metastasis with colorectal carcinomas. In flow cytometric analysis, CD4+ and CD8+ T lymphocytes, macrophages and myeloid-derived suppressor cells (MDSCs), which are easy to obtain in the peritoneal cavity, were revealed to have significant differences between immunized and non-immunized mice and these contributed to antitumor responses. We also observed that both serum and peritoneal lavage fluid harvested from immunized mice showed the presence of CT26-specific autoantibodies. In addition, increase in level of TGF-β1 and IL-10 in serum but a decrease of TGF-β1 in peritoneum was found. Taken together, these findings may provide a new vaccine strategy for the prevention of peritoneal and even systemic metastasis of carcinomas through induction of an autoimmune response in the peritoneum.

Keywords: Peritoneum - immune response - colorectal carcinoma - metastasis

Introduction

The leading cause of cancer related mortality is metastatic spread of tumor cells to vital organs. Peritoneal metastasis and carcinomatosis is the most common secondary cancerous disease to affect the peritoneal cavity for patients with cancers such as gastric, hepatic, ovarian and colorectal carcinoma, implying poor prognosis. Standard therapy used to be a cytoreductive surgery in combination with adjuvant chemotherapy. To improve the therapeutic outcome, developing a strong antitumor immunization before metastasis is therefore essential for metastatic prevention and therapeutic strategies assisting for local tumor.

Metastasis is a multistep process comprising intravasation, survival in the circulation, extravasation, and colonization of distant tissues (Chambers et al., 2002; Joyce et al., 2009), and the peritoneal dissemination and carcinomatosis processes consist of local inflammatory reaction, changes in the expression of adhesion molecules, proteolytic enzymes, and growth factors, destruction of peritoneum, attachment of carcinoma cells and colonization. The peritoneal cavity is proved to be home to a specific type of macrophage (Cailhier et al., 2005; Liu et al., 2006), rich effector memory T lymphocytes (Roberts et al., 2009), a resident population of self-renewing B lymphocytes (Hardy et al., 1994; Paciorkowski et al., 2000), a new subset of tissue-resident NK cells (Gonzaga et al., 2011) and a newly described population found in fat-associated lymphoid clusters (Moro et al., 2010). These cell populations play a critical role in initiating acute peritoneal inflammation and host protection. Not much is known, however, about the alteration in quantity and function of the resident cell populations to produce autoimmune response for tumor in peritoneum. We speculate that these cells form part of the first line of defense against invading pathogens and make peritoneal to be a microenvironment, apart from subcutaneous, which could develop strong autoimmunization response against tumor metastasis in peritoneum and even systemic anti-tumor effect.

To test this concept, we describe the accumulation and quantification of T lymphocytes, B lymphocytes, macrophages, and (myeloid-derived suppressor cells) MDSCs by the primary tumor after peritoneal immunization in mouse cancer models, and observe the anti-tumor effect by i.p. immunization. In the study, the irradiated tumor cells as vaccine by i.p. injection is shown to be effective in both peritoneal and systemic antitumor immunity, in which autoreactive immunity may be directed against the tumor cells in both humoral and cellular immunity. These observations may provide a new vaccine strategy for the prevention of peritoneal and systemic metastasis of carcinomas through the induction of the autoimmune response in peritoneum, in addition to subcutaneous immunization.
Materials and Methods

Mice and cell lines

Five- to six-week-old female BALB/c and C57BL/6 mice were purchased from Vital River and housed in microisolators until the age of 8 weeks before using them in experiments. CT26 colorectal carcinoma cell line, B16 melanoma cell line, and EG7 lymphoma cell line are used in this study. Colorectal cancer and lymphoma cells were maintained in RPMI medium 1640 with 10% FBS. Melanoma cells were grown in DMEM supplement with 10% FBS. CT26 colorectal cancer models were established in BALB/c mice. B16 melanoma and EG7 lymphoma models were established in C57BL/6.

Immunization and peritoneal lavage fluid harvesting

Mice were immunized by peritoneal injection of irradiated tumor cells or saline alone (nonimmunized mice) for 3 times at day 0, 14, and 21, and challenged with tumor cells i.p. at day 28. According to the time point, the peritoneal cavity was lavaged with 5ml PBS for 2 times after sacrificing the mice immediately. The 10ml fluid was next centrifuged at 2000 rpm for 4 minutes and kept as single-cell suspensions in 1ml PBS for flow cytometric analysis and lavage supernatant for antibody and cytokine production evaluation, respectively.

Flow cytometric analysis

Peritoneal lavage samples from groups of immunized and nonimmunized mice were pooled, spun, and resuspended in 1 ml of sterile PBS. Expression levels of Gr1, CD11b, F4/80, CD4, CD8, CD11c, MHC-II and CD69 on these lavage cells labeled with fluorescence-conjugated antibodies (BD PharMingen) and isotype-matched IgG controls were analyzed by flow cytometry (BD FACS Calibur). To assay the presence of CT26-specific autoantibodies, tumor cells were stained by an indirect method (Wei et al., 1996; Wei et al., 2000) using 1:50 to 3000 diluted serum or peritoneal lavage supernatant, and then FITC-goat anti-mouse IgG, IgM, and IgA (Sigma-Aldrich).

Cytokine response

Mice were immunized for 3 times arranged at day 0, 14, and 21 and underwent peritoneal lavage before or following the i.p. injection of CT26 tumor cells. Lavage fluid was centrifuged, aliquoted, and stored at −80°C until analyzed by ELISA for cytokine. Serum was also harvested at the same time from immunized and control groups and evaluated for the production of TGF-β and IL-10 by ELISA (RB).

Statistical analysis

For comparison of individual time points, ANOVA and an unpaired Student t test were used for our data analysis statistically (Sauter et al., 2000). Statistical significance was determined by the log-rank test (Peto et al., 1972). Error bars were from standard deviations, and p values less than 0.05 were considered significant. Survival curves were constructed according to the Kaplan-Meier method (Kaplan et al., 1958).

Results

Effective antitumor immunity of peritoneal cavity

To investigate the protective antitumor immunity, we immunized mice (10 mice in each group) by the s.c. or i.p. injection of irradiated CT26 colorectal carcinoma or saline alone (nonimmunized mice) for 3 times arranged at day 0, 14, and 21, and then challenged mice with CT26 cells at day 28 s.c. or i.p., respectively. As shown in Figure 1, tumors grew progressively in all nonimmunized mice by s.c. inoculation (saline alone) (Figure 1A) and the survival...
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Figure 3. Changes of Cell Population in Peritoneal Following Engraftment in Two Groups. Mice were immunized i.p. as described before and challenged with 5x 10^5 live CT26 cells i.p.. The diversification of the immune cell composition in peritoneum was analysed at different time point (before the injection, 4h, 24h, and 4d later) for immunized and nonimmunized group respectively. A, B, C and D, Flow cytometry analysis for CD4+ CD8+ T cells, F4/80+CD11b+ macrophages and Gr-1+CD11b+ MDSCs in peritoneal upon tumor cell challenge of BABL/c mice over time (left panel). Quantification of cells is presented in the right panel (n=5).

Cellular immune response in peritoneum induced by the irradiated tumor cells

In an attempt to explore the possible mechanism by which the antitumor activity was induced with irradiated tumor cells, we investigated the differences of the immune cell composition in peritoneum that could influence dissemination and metastasis. We stained the cell populations at different time point (before the injection, 4h, 24h, and 4d later) for two groups, and found that a small amount of CD4+ (Figure 3A) and CD8+ T (Figure 3B) lymphocytes exist in the peritoneal of mice in two groups, decrease after the i.p. inoculation in nonimmunized group, but maintain nearly the same percentage in immunized group, suggesting that T cells may be required for the antitumor response. Besides, the amount of CD4+T lymphocytes (1.3%-20.3%) is always higher compared to CD8+T lymphocytes (0.45%-8.08%) in both immunized or nonimmunized mice. No differences in numbers of CD19+B lymphocytes (34.4%-39.9%) could be identified between two groups by flow cytometry. However, immunized mice displayed reduced numbers of F4/80+CD11b+ macrophages (4.94%-16.6%) but a obvious increase in nonimmunized group was shown (24.9%-47.9%) (Figure 3C), suggesting that the macrophages may be no longer needed or less but more active for antitumor activity. In addition, we investigated whether myeloid cell populations are recruited to CT26...
cells in peritoneal. Significant association of Gr-1\(^+\)CD11b\(^+\)
cells (MDSCs) with the injection of CT26 colorectal
tumor cells into peritoneal was observed shortly after
the treatment. There is only a small amount of MDSCs in
the peritoneal of immunized or nonimmunized mice(0.89%-
2.05%). However, distinguished increase showed up 4h
later after the inoculation (18.7%-28.4%) but decreased
over time (Figure 3D). Quantification of the MDSCs
revealed that the nonimmunized mice could recruit larger
amount cells than immunized ones, indicating that the
recruit may be inhibited and MDSCs are needed for the
first inflammatory response.

Autoantibodies both in sera and peritoneal and their
systemic antitumor efficacy

Mice were immunized in peritoneal three times
as before. Serum and peritoneal lavage supernatant
harvested from immunized mice at day 28 were assayed
for the presence of CT26-specific autoantibodies in flow
cytometric analysis. Tumor cells were stained with sera
or peritoneal lavage supernatant isolated from immunized
and nonimmunized mice. As a result, tumor cells showed
the positive staining both with sera or supernatant from
immunized mice, but negative staining for unimmunized
mice (Figure 4A-B).

The systemic antitumor efficacy induced by irradiated
tumor cell was next tested. Mice were immunized
in peritoneal and then challenged with CT26 cells on day
28 s.c.. Tumor growth in all mice by s.c. inoculation was
recorded (Figure 4C). Tumor nodules were only turned
out in two mice (20%) until day 17 in immunized group,
while they got a 100% incidence in unimmunized mice
(Figure 4D), suggesting that intraperitoneal immunization
could induce an effective systemic antitumor efficacy.

Cytokine response

Serum and peritoneal lavage supernatant harvested
were evaluated for the production of TGF-β1 and IL-10
by ELISA. As shown in Figure 5A-B, the increase in the
level of TGF-β1 and IL-10 was found in the serum when
compared with the controls. The lavage fluid supernatant
from immunized mice only got a weak increase of IL-10
and a decrease of TGF-β1.

Discussion

Peritoneal cavity is a microenvironment with
rich resident and specific cells and surrounded by
profuse arteriovenous network system. Yet its immune
function has not been identified. In the present study
several observations have been made concerning the
immunization vaccine by i.p. injection, autoimmune
response, and antitumor effection. The result, to our
knowledge, first demonstrated that the microenvironment
of peritoneal cavity could develop a strong peritoneal
and systemic antitumor immunization effect by i.p. injection
of normal irradiated tumor cell vaccines in mouse
cancer models. Furthermore, our findings suggest that
autoreactive immune response in peritoneum against the
tumor cells may be provoked by the alteration in quantity
and function of the resident cell populations such as T
cells, macrophages and MDSCs, and that the antitumor
activity following i.p. vaccination may be involved in
both T cell effectors and autoantibodies. Both CD4\(^+\)
and CD8\(^+\) T cells showed more stable and higher level in
immunized mice. Macrophages and MDSCs found to be
less in percentage. Autoantibodies in sera, peritoneum
and on the tumor cells were identified. IgG subclasses
were substantially increased in response to vaccine by i.p.
injection. The inhibition of the in vivo tumor growth by
s.c. inoculation was found when the vaccine was injected
i.p.. These findings suggest that humoral immunity may be
responsible for the antitumor activity by the vaccination.

We found in the present study that in vivo immunized
mice have lower level of CD4\(^+\) T lymphocytes and higher
CD8\(^+\) T lymphocytes comparing to normal mice. But these
cell populations could keep the level after peritoneal tumor
cells inoculation in immunized mice, while the decrease
of T lymphocytes was found in nonimmunized mice.
These findings suggest that T cells may be required for
the antitumor response, and these suggestions were further
supported by the important roles of CD4\(^+\) T lymphocytes
in the antitumor immunity (Bennett et al., 1997; Carbone
et al., 1998; Hung et al., 1998; Ossendorp et al., 1998;
Pardoll et al., 1998; Toes et al., 1999; Hu et al., 2000).
Besides, it has been reported that CD4\(^+\) T lymphocytes
are required for the generation and maintenance of
cytolytic CD8\(^+\) T cells (Pardoll et al., 1998; Hu et al.,
2000) and believed to be essential for the developing of
both a cellular and humoral antitumor immune response
(Bennett et al., 1997; Hung et al., 1998; Ossendorp et al.,
2000).
M2 macrophages express a wide array of anti-inflammatory molecules, such as IL-10 and TGF-β1. TAMs also release growth factors such as VEGF, transforming growth factor β (TGF-β), and a member of the FGF family, to promote angiogenesis in many tumors (Tanaka et al., 2002; Siveen et al., 2009; Solinas et al., 2009). Transforming growth factor-β1 was proved to facilitate cell adhesion to ECM growth factors such as VEGF, transforming growth factor (Tanaka et al., 2002; Siveen et al., 2009; Solinas et al., 2009). Transforming growth factor-β1 was proved to facilitate cell adhesion to ECM growth factors such as VEGF, transforming growth factor (Tanaka et al., 2002; Siveen et al., 2009; Solinas et al., 2009). Transforming growth factor-β1 was proved to facilitate cell adhesion to ECM growth factors such as VEGF, transforming growth factor (Tanaka et al., 2002; Siveen et al., 2009; Solinas et al., 2009). Transforming growth factor-β1 was proved to facilitate cell adhesion to ECM growth factors such as VEGF, transforming growth factor (Tanaka et al., 2002; Siveen et al., 2009; Solinas et al., 2009). Transforming growth factor-β1 was proved to facilitate cell adhesion to ECM growth factors such as VEGF, transforming growth factor.
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