Relationship between the expressions of PD-L1 and tumour-associated fibroblasts in gastric cancer

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ABSTRACT
Previous studies have focused on the changes of tumour cells in immune escape, and less is known about the effect of tumour microenvironment (TME) on immune escape. Tumour-associated fibroblasts (TAFs) are an important part of the TME and have special physiological and biochemical characteristics, but the specific mechanism has not been clarified. In order to investigate the effect of TAF on the expression of PD-L1 in gastric cancer cells, gastric cancer cell lines MNK45, SGC7901 were non-contact co-culturing with TAF 1, 3 and 7 d via transwell. PD-L1 mRNA and protein expression were detected using qRT-PCR and FCM. Then, 95 cases of gastric cancer tissues were selected and evaluated PD-L1 and TAF expressions by immunohistochemical examination. The results showed that the mRNA and protein expression of PD-L1 in the experiment group were significantly higher than that in the control group. PD-L1 expression was associated with massive lymphocyte infiltration, diffuse/mixed histology and intratumoral TAFs in gastric cancers. In conclusion, TAFs promoted the growth in gastric cancer cell lines by increased the PD-L1 expression.

Introduction
Gastric cancer (GC) is one of the world’s most common digestive system malignant tumour, especially in East Asian countries such as South Korea, Japan and China [1–3]. The prognosis for advanced-stage GC is grim, even with the use of chemotherapy or other adjuvant treatments [4–6]. Tumour cells can escape from the immune surveillance. Previous studies have focused on the changes of tumour cells in immune escape, however, little is known about the effect of tumour microenvironment (TME) on immune escape [7–9]

Tumour-associated fibroblasts (TAFs) are the most important host cells in the microenvironment stroma, which take part in extracellular matrix construction and cancer colony development [10,11]. Compared with normal tissue fibroblasts, TAFs have significantly different morphology and protein expression patterns [12]. For instance, TAFs express α-SMA and vimentin and secrete TGF-β, PDGF, FGF, VEGF, IL-11, IL-6 and extracellular matrix components, which can promote cancer cell growth, induce tumour angiogenesis and enhance metastasis and invasion [13–15]. It is now clear that there is continual crosstalk not only between tumour cells and immune cells but also between other stroma cells such as TAFs and immune cells. Because of their preponderance in the tumour microenvironment, TAFs have recently been investigated for their function as a regulator of immune cell recruitment and function.

Programmed death factor 1 (PD-1) and its ligands PD-L1/ PD-L2 are negative regular factor, which play a very pivotal role in tumour escape host immune response [16–20]. Expressions of PD-L1 in cancer patients are associated with depth of invasion, lymphatic metastasis, pathological pattern and overall survival. PD-L1 monoclonal antibody in melanoma and non-small cell lung cancer and other tumours have a good curative effect but poor curative effect on the digestive system tumours [21–23]. Recently, PD-L1 has been reported to be expressed in TAFs [24]. TAFs can influence tumour growth by producing growth factors and chemokines [25,26].

To elucidate the effect of tumour-associated fibroblasts in tumour microenvironment on the immunological properties of tumour cells, we observed the expression of the immunosuppressive molecule PD-L1 by constructing the co-culture system of gastric cancer cell lines and gastric cancer-related fibroblasts and then relationships between clinicopathological parameters of GC and PD-L1 expression associated with TAFs.

Materials and methods

Tissue samples and cell lines
Ninety-five gastric cancer tissues and adjacent normal tissues which had not treated with radiation therapy or chemotherapy before surgery were obtained from Department of
General Surgery, Qingdao University. All these tissues were immediately snap-frozen in liquid nitrogen after surgery. The study was approved by the Ethics Committee of Yuhuangding Hospital (2016081206).

**Cell culture**

Human gastric cancer cell lines (SGC7901 and MNK45) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco modified Eagle medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), 100 U/mL penicillin and 100 ng/mL streptomyacin in condition of at 37 °C temperature with 5% CO₂ and 95% air.

Human gastric TAFs were derived from surgical tissue of 95 patients with GC. TAFs were established from the tumour gastric wall, and normal fibroblasts (NFs) were from non-tumoral gastric wall. The fresh specimens were washed three times with PBS containing 100 U/mL penicillin and 100 μg/mL streptomyacin and cut into small pieces and digested with collagenase I. Then the mixture was centrifuged and rinsed. The cells with PBS, the pellets were re-suspended in DMEM supplemented with 10% FBS and transferred to 100 mm dishes. These cell lines cultured in RPMI 1640 or DMEM containing 10% fetal bovine serum (FBS). For the co-culture assays, the cell lines were cultured in RPMI 1640.

**Cell co-culture model**

Human gastric cancer cells (SGC7901 and MNK45) and TAFs were non-contact co-cultured *in vitro* via transwell (Corning Incorporated Life Science, Acton, MA, USA). Two chambers were separated by a semi-permeable membrane with a pore size of 0.4 μm and 4.5 cm² area (BD Biosciences). Human gastric cancer cells (SGC7901 and MNK45) were cultured at the density of 3 × 10⁵ cells/mL in the upper chambers of the transwell insert. The lower chambers were cultured in TAFs cells at the density of 5 × 10⁵ cells/mL or contained normal fibroblasts at the density of 5 × 10⁵ cells/mL (for controls).

**Cell proliferation assays**

Cell proliferation was evaluated by the alamarBlue assay. After culture for 1, 3 and 7 d, the culture medium was replaced by Medium 1640 (GIBCO, Carlsbad, CA, USA) supplemented with 10% FBS and 0.1% NaN₃ twice. After incubating with alamarBlue-labelled or isotype control antibodies for 30 min at 4 °C, cells were washed with FCM buffer and analyzed on a Beckman coulter FC500 with submit5.2 software (Beckman Coulter, USA).

**PD-L1 protein expression**

The expressions of PD-L1 were analyzed with FCM in experimental and control groups. The co-cultured or control cultured cells were harvested and washed with FCM buffer (PBS with 5% FBS and 0.1% NaN₃) twice. After incubating with fluorescein labelled or isotype control antibodies for 30 min at 4 °C, cells were washed with FCM buffer and analyzed on a Beckman coulter FC500 with submit5.2 software (Beckman Coulter, USA).

**Immunohistochemical staining**

Immunohistochemistry was performed according to a conventional protocol [27]. The paraffin-embedded specimens were sectioned at a thickness of 4 μm and incubated in a dry oven at 63 °C for 1 h. De-paraffinization and rehydration were then performed. The sections were incubated with the following primary antibodies: polyclonal rabbit anti-human PD-L1 (clone ab82059, abcam, UK; 1:100), monoclonal mouse anti-human α-SMA (clone 4B12, Dako, Denmark; 1:80) were treated for 1 h at room temperature. Negative controls were performed by replacing the specific antibody with mouse IgG isotype (Sigma, St. Louis, MO, USA).

**Statistical analysis**

All statistical analysis was carried out using the SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). All values are expressed as the mean ± standard deviation from at least three repeated individual experiments for each group. The significant differences were analyzed using Student’s t-test analysis for difference between two groups, and person’s chi-square test and Fisher’s exact test were used to detect the correlation between PD-L1 expression and other clinicopathological characteristics. Differences were considered significant when p < .05.

**Results**

**Primary culture and characteristic of TAFs and NFs**

The fibroblast markers, fibronectin (FN) and α-smooth muscle actin (SMA) were determined by immunofluorescence staining. The results showed that FN positive expression in TAFs and NFs cells. Moreover, α-SMA was high expression in TAFs and a biomarker for myofibroblasts (Figure 1). Further analyze showed that gastric cancer cells co-cultured with TAFs grew faster compared with the control groups (Figure 2).

**The PD-L1 expression in gastric cancer cells**

We further assessed the effects of PD-L1 expression with qRT-PCR and FCM in the gastric cancer cell lines co-cultured
with TAFs and the control groups. The results revealed that the mRNA expression of PD-L1 was markedly increased in gastric cancer cell lines co-cultured with TAFs in a time-dependent manner. Similarly, the protein expression of PD-L1 exhibited similar results (Figure 3).

The expression of PD-L1 in gastric cancer tissues

The expression levels of PD-L1 in gastric cancer tissues were evaluated and its relationship with clinical and pathological of GC patients was analyzed. There was no significant difference between PD-L1 levels and gender (\(p = .845\), Table 1), tumor site (\(p = .123\), Table 1), tumor invasion (\(p = .634\), Table 1) and metastasis (\(p = .476\), Table 1) and tumor-node-metastasis (TNM) staging (\(p = .675\), Table 1). There was a positive correlation between PD-L1 expression and younger age (\(p = .023\), Table 1) and inverse correlation between PD-L1 expression and tumour nodal status (\(p = .035\), Table 1).

Relationships between PD-L1 expression and densities of TAFs

TAFs were positive for PD-L1 in 53 cases (82%) and stained in a scattered-dot pattern within the cytoplasm. The density of intratumoral TAFs showed a significant positive correlation with the PD-L1 expression of tumour cells (\(p = .027\), Table 1). TAFs were also positively correlated with age (\(p = .013\), Table 2) and TNM staging (\(p = .025\), Table 2).

Discussion

Gastric cancer is one of the world’s most common digestive system malignant tumour, especially in East Asian countries such as South Korea, Japan and China. Tumour cells can escape from the immune surveillance. Previous studies have focused on the changes of tumour cells in immune escape, and less is known about the effect of tumour microenvironment (TME) on immune escape [28–30].

In early stages of cancer development, various types of cells in tumour stroma prevent tumour growth. In contrast, most of these stromal cells promote tumour progression in advanced stages [31]. TAFs have played a critical role by secreting various soluble factors [32–34]. It has previously revealed that the prevalence of TAFs was closely correlated with the potential of gastric cancer metastasis [35]. Moreover, TGF-β and FGF-7 produced from fibroblasts could increase gastric cancer cell proliferation and invasiveness [36,37]. Several reports have also demonstrated the TAFs express MCP-1/CCL2 when co-cultured with cancer cells and induce cell survival and immunosuppression [26,38]. Together relevant results suggested that TAFs were first educated by immune cells during the initial stages of tumorigenesis but, as tumour progression occurs, they acquire the ability to recruit and regulate immune cells to an eventually immune-suppressed which is compatible with disease progression. However, factors important for cancer immune escape in direct interactions between TAFs and cancer cells remain unknown.

Immunological resistance represents one of the major obstacles for effective tumour biotherapeutic options for a wide variety of human cancers. PD-L1, a key protein located in the tumour microenvironment, may promote the immune escape of tumour cells [39]. It is now well known that PD-L1 over expression in cancer cells could induce tumour evasion from the host T cells [40,41]. Blockade of PD-1/PD-L1 interaction or down-regulation of PD-L1 expression in cancer cells has been reported to promote anti-tumour immunity and inhibit tumour growth [41]. The regulation of PD-L1 expression remains largely unknown. In the present study, we report that TAFs co-cultured could able to up-regulate PD-L1 expression in human gastric cancer cell lines in a time-dependent manner and induced the cancer cells proliferation. The density of intratumoral TAFs showed a significant positive correlation with the PD-L1 expression of tumour cells (\(p = .027\)). Combined with previous results, this suggests that the induction of PD-L1 expression by TAFs in cancer cells is a common effect. Previous studies showed that immunohistochemical PD-L1 expression was strongly positive in 42.2% of 102 human gastric carcinomas, weakly positive in adenoma samples and totally negative in normal gastric tissue [42]. Moreover, PD-L1 expression was significantly correlated to a higher number of lymph node metastasis, larger tumour size, increased depth of invasion and poorer overall survival. Same results were presented that one of the anti-PD-L1 mAbs developed during the investigation significantly reduced the T-cells apoptosis induced by PD-L1 overexpression [43].

Several clinical trials have demonstrated that blocking the PD-1/PD-L1 pathway with a PD-L1 antibody results in antitumor effects [44,45]. With higher grade, tumour cells overexpress PD-L1 in infiltrating ductal carcinoma (\(p = .012\)) [46], urothelial carcinoma (\(p < .001\)) [29], and intrahepatic
cholangiocarcinoma ($p < .001$) [29]. In our study, the expression of PD-L1 is significantly associated with younger age ($p = .023$) and diffuse/mixed type ($p = .032$). There was an inverse correlation between PD-L1 expression and tumour nodal status ($p = .035$). However, no statistical relationships were found between the expression of PD-L1 and gender, tumour site, tumour invasion and metastasis and TNM staging. The probable reason is the relatively small number of PD-L1 positive cases. Similar results showed that no statistical relationships were found between the expression of PD-L1 and clinical stage/survival rate in non-small cell lung carcinomas [47,48].

In normal human body, PD-1 and CD28 are in dynamic balance, but PD-1 and its ligand are high expressed in tumour patients [46]. Tumour cells with high expression of PD-L1 inhibited the proliferation and function of CD8+ T cells. Meanwhile, high expression of PD-L1 also inhibited the proliferation and function of NK cells and B cells [49]. When stimulated by PD-L1, Treg cells can enhance their ability to proliferation and up-regulate their functions [50]. All of the above-mentioned findings lead to tumour escape from immune surveillance and promote tumour growth. Therefore, the underlying mechanisms should be explored in the future to fully elucidate the association of PD-L1 with the prognosis of GC patients.

In conclusion, we have demonstrated that the expression of PD-L1 was markedly increased in gastric cancer cell lines co-cultured with TAFs in a time-dependent manner.
Figure 3. The PD-L1 mRNA and protein expression in SGC7901 cell (A,C) and MNK45 cell (B,D). The expression of PD-L1 of gastric cancer cells when co-cultured with TAFs were significantly increased in a time-dependent compared with the control groups.

Table 1. Correlation between PD-L1 expression and clinicopathological factors.

| Characteristics      | N | PD-L1 (+) | PD-L1 (-) | p  |
|----------------------|---|-----------|-----------|----|
| Gender               |   |           |           |    |
| Male                 | 57| 37        | 20        | .845|
| Female               | 38| 28        | 10        |    |
| Age                  |   |           |           |    |
| ≤60                  | 50| 40        | 10        | .023*|
| >60                  | 45| 25        | 20        |    |
| Tumour site          |   |           |           |    |
| Antral               | 60| 42        | 18        |    |
| Proximal             | 35| 23        | 12        |    |
| Invasion             |   |           |           |    |
| T1                   | 25| 18        | 7         |    |
| T2                   | 20| 17        | 3         |    |
| T3                   | 26| 13        | 13        |    |
| T4                   | 24| 17        | 7         |    |
| Nodal status         |   |           |           |    |
| N0                   | 42| 26        | 16        | .035*|
| N1                   | 13| 9         | 4         |    |
| N2                   | 21| 15        | 6         |    |
| N3                   | 19| 15        | 4         |    |
| Metastasis           |   |           |           |    |
| Yes                  | 5 | 4         | 1         | .476|
| No                   | 90| 61        | 30        |    |
| TNM staging          |   |           |           |    |
| I                    | 32| 25        | 7         | .675|
| II                   | 26| 17        | 9         |    |
| III                  | 30| 17        | 13        |    |
| IV                   | 7 | 6         | 1         |    |
| Lauren classification|   |           |           |    |
| Intestinal type      | 40| 25        | 15        | .032*|
| Diffuse/mixed type   | 55| 40        | 15        |    |
| Tumour-associated fibroblasts | | | | .027*|
| Low                  | 30| 12        | 18        |    |
| High                 | 65| 53        | 12        |    |

Table 2. Correlation between PD-L1 expression and density of ATFs.

| Characteristics      | N | PD-L1 on tumor-associated fibroblasts | p  |
|----------------------|---|--------------------------------------|----|
| Gender               |   |                                       |    |
| Male                 | 37| 17                                   | 20 |
| Female               | 28| 18                                   | 10 |
| Age                  |   |                                       |    |
| ≤60                  | 40| 30                                   | 10 |
| >60                  | 25| 5                                    | 20 |
| Tumour site          |   |                                       |    |
| Antral               | 42| 22                                   | 20 |
| Proximal             | 23| 13                                   | 10 |
| Invasion             |   |                                       |    |
| T1                   | 18| 10                                   | 8  |
| T2                   | 17| 7                                    | 10 |
| T3                   | 13| 7                                    | 6  |
| T4                   | 17| 11                                   | 6  |
| Nodal status         |   |                                       |    |
| N0                   | 26| 12                                   | 14 |
| N1                   | 9 | 7                                    | 2  |
| N2                   | 15| 7                                    | 8  |
| N3                   | 15| 9                                    | 6  |
| Metastasis           |   |                                       |    |
| Yes                  | 4 | 2                                    | 2  |
| No                   | 61| 33                                   | 28 |
| TNM staging          |   |                                       |    |
| I                    | 25| 20                                   | 5  |
| II                   | 17| 9                                    | 8  |
| III                  | 17| 3                                    | 14 |
| IV                   | 6 | 3                                    | 3  |
| Lauren classification|   |                                       |    |
| Intestinal type      | 25| 10                                   | 15 |
| Diffuse/mixed type   | 40| 25                                   | 15 |

*p < .05.
PD-L1-positive tumour cells were found in most GC patients (68%), and which were associated with the densities of TAFs (82%). Furthermore, our study found that PD-L1 expression was associated with massive lymphocyte infiltration, diffuse/mixed histology and intratumoral TAFs in GC. Taken together, blockade of tumour PD-L1 is expected to enhance the effects of immunotherapy.

**Ethics approval and consent to participate**

The present study was approved by the Institutional Animal Care and Use Committee of Shandong University (Jinan, China).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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