Evaluation of immune response according to the metastatic status in the regional lymph nodes in patients with gastric carcinoma

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Abstract. The immunological response in the regional lymph nodes (LN) of gastric cancer patients who had metastasis was studied by investigating both the degree of metastasis and the maturation status of dendritic cells (DCs). A total of 732 LNs was obtained from 29 gastric cancer patients, including 25 patients whose LNs were pathological metastasis-negative and 4 patients with metastasis-positive LNs. Micrometastasis (MM) in the LNs was analyzed by immunohistochemical staining of cytokeratin (CK) (IHC-MM) and by amplifying CEA and CK19 mRNA by quantitative real-time RT-PCR (PCR-MM). Distribution and density of mature DCs were evaluated in 119 LNs from pathological metastasis-positive cases by examining CD83 expression immunohistochemically. Then, following the examination of PCR-MM, immunological responses were analyzed by amplifying CD83, CD86, CD1a and IFN-γ mRNA by quantitative real-time RT-PCR in 613 LNs from 25 pathological metastasis-negative cases. Among 119 LNs from 4 patients with LN metastasis, 20 LNs had histological metastasis and 6 histological metastasis-negative LNs had IHC-MM. The distribution and density of mature DCs were identical in the LNs regardless of metastasis status. Among 613 LNs from 25 patients without histological LN metastasis, 15 LNs (2.45%) from 6 cases (24%) were PCR-MM positive. The expression levels of CD83 and CD86 mRNA were significantly higher in the 15 PCR-MM-positive LNs than in the 598 PCR-MM-negative LNs. The expression levels of CD83 and CD86 mRNA in 136 PCR-MM-negative LNs from 6 PCR-MM-positive cases were also higher than those in 462 LNs from 19 PCR-MM-negative cases. In gastric cancer patients, activation of the immune response by maturation of DCs is wide-spread in regional lymph nodes at a stage before metastasis is detected histologically, including detection by IHC-MM.

Introduction

Dendritic cells (DCs) function as professional antigen-presenting cells (APCs) and are important in the initiation of primary immune responses (1). The function of DCs is highly influenced by their level of maturation, and in the T-cell-driven response to tumor presentation the maturations of DCs and T cells are thought to be instrumental.

DCs are heterogeneous in regard to maturation, differentiation and state of activation, (2) which are controlled and regulated by a variety of microenvironmental signals, including cytokines and surface molecules expressed on neighboring cells (3,4). In most tissues, DCs are present in a so-called ‘immature’ state and are able to capture tumor-specific antigens, when they encounter apoptotic or necrotic tumor cells in the primary tumor. Afterwards, DCs process the antigens into peptides that are presented in the context of MHC class I and class II molecules and migrate from the primary tumor to the regional LNs, where they interact with naïve T-cells and activate naïve T-cells and change them to antigen-specific T-cells. CD1a, a cell surface glycoprotein that is structurally related to the MHC molecules and mediates an MHC-independent antigen presentation pathway, is a highly specific and sensitive marker of immature DCs and Langerhans cells, a population of DCs found in the skin (7,8). CD83 is a glycoprotein member of the immunoglobulin superfamily that is strongly up-regulated during DC maturation, thus making it a robust marker for mature DCs and Langerhans cells, a population of DCs found in the skin (7,8). CD83 is a glycoprotein member of the immunoglobulin superfamily that is strongly up-regulated during DC maturation, thus making it a robust marker for mature DCs (9-12). Activation of naïve T-cell by mature DCs requires two signals. The first signal is transmitted via the MHC molecule/T cell receptor for the recognition of tumor-specific antigen by DCs (13) but is insufficient to induce the immune response. The second signal is required for T-cells to acquire the functions of an effector cell (14) and it is provided by costimulatory molecules such as CD86 and CD80, which are expressed on mature antigen-presenting cells (APC), including the DCs, and bind to the ligands of costimulatory molecules that are expressed on T cells (15,16). In the absence of a costimulatory signals, T cells typically enter a state of anergy.

IFN-γ is one of the T helper 1 (Th1) types of cytokines, and its production is inducible by signals from the T-cell receptor and co-stimulatory molecules in the presence of...
IL-12. DCs secrete IL-12, which promotes naïve T-cells to differentiate into IFNγ-secreting Th1 cells (17,18).

Current immunological studies have demonstrated that dense infiltration of a variety of malignant tumors by DCs correlates with a favorable prognosis, suggesting that DCs play a critical role in an anti-tumor response (19). A high degree of DC infiltration into the primary tumor was reported to reflect the host immune defense mechanism (3). Similarly, in gastric cancer, DC infiltration into the primary tumor was reported to decrease the progression of nodal involvement and lead to good survival rates (20-25). Lymph node metastasis is a crucial factor that influences prognosis, and patients with nodal involvement in gastric cancer have a poor clinical course compared with those without metastasis (26). However, the clinical significance of micrometastases (MM) still remains controversial. In the 6th edition of the TNM classification, micrometastases were classified as MM or isolated tumor cells (ITC) according to their largest dimensions. Some authors have reported a significant correlation between the presence of MM and prognosis in gastric cancer (27-29), whereas others have not (30,31). Morgagni et al (27) defined MM as single cells or small clusters of neoplastic cells identifiable by immunohistochemical methods and concluded that the presence of MM in early gastric cancer (pN0) does not influence patient prognosis. Fukagawa et al (30) also defined MM as the presence of tumor cells detected by cytokeratin-specific immunohistochemistry and not by ordinary HE staining. However, they concluded that the immunohistochemical presence of MM in the regional lymph nodes does not affect the survival of gastric cancer patients. This study had some problems regarding the different antibodies used, the detection technique, the objects and the definition of MM. The main concern was whether a small number of cancer cells dissociated from the primary tumor can survive after being trapped in the regional LNs. Mature DCs activated in the primary tumor might play a role as self-immune defense mediators in the regional LNs. Tsujitani et al (32) suggested that DCs in the regional LNs are important for establishing immunological defense mechanisms in cases of gastric cancer. The relation between the extent of LN metastasis, especially MM, and the immune response based on the maturation status of DCs in the regional LNs in gastric cancer patients is still uncertain.

In the present study, we aimed to investigate the relation between immune response and metastasis status in the regional LNs by examining the maturation status of DCs and degree of LN metastasis in gastric cancer patients.

**Materials and methods**

**Patients.** We studied 731 LNs from 29 gastric cancer patients who underwent gastrectomy at the Department of Gastroenterological Surgery, Kanazawa University Hospital from April 2003 to 2005, with informed consent. None of the patients had received preoperative chemotherapy or radiotherapy. Our study includes 4 patients with LN metastasis and 25 patients without LN metastasis, which was diagnosed by routine histological examination using haematoxylin and eosin (H&E) staining. Four patients with LN metastasis underwent total or partial gastrectomy with more than D2 LN dissection. A total of 25 LN-negative patients underwent gastrectomy with lymphatic mapping and lymphatic basin dissection (33) followed by segmental gastrectomy in 11 cases, local gastrectomy in 2 cases, proximal gastrectomy in 2 cases and distal gastrectomy in 10 cases. The clinicopathological features of the 25 patients without LN metastasis are classified according to the Japanese Classification of Gastric Carcinoma (13th edition) and shown in Table I.

**Cancer cell line and normal control LNs.** The human gastric cancer cell line MKN45 was used as a positive control for cancer cell detection by a quantitative RT-PCR assay. The cell line was maintained in RPMI-1640 (Nissui Pharma Co., Ltd., Tokyo, Japan) supplemented with 10% heated, inactivated fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD, USA) at 37°C and 5% CO₂. As a negative control, 22 normal lymph nodes from non-cancerous patients (chronic cholecystitis, ulcerative colitis, diverticulitis and hepatic artery aneurysm) were obtained at the time of surgery (cholecystectomy, partial colectomy, resection of aneurysm) with their
informed consents and studied in order to determine the cut-off value of the quantitative RT-PCR assay.

**Distribution and density of mature DCs in the regional LNs in pathological metastasis-positive patients.** A total of 119 LNs from 4 LN-positive gastric cancer patients were assessed by immunohistochemistry for the distribution and density of DCs according to the degree of metastasis. All LNs dissected from the patients were cut into halves at the plane of the largest dimension. One half of each LN was fixed in 10% buffered formalin and embedded in paraffin for histological examination. The other half was immediately embedded in O.C.T. (Tissue-Tek Optimal Cutting Temperature) compound (Sakura Finetek, Tokyo, Japan), frozen and stored at -80°C until immunohistochemical analysis. Each frozen LN was cut into 6-μm slices and incubated with the primary antibody, rabbit polyclonal anti-keratin, (1:600) for 1 min at room temperature and mouse monoclonal anti-CD83 (clone sc-19679, Santa Cruz Biotechnology Inc., Santa Cruz, CA; dilution 1:100) for 4 min at room temperature in order to detect small clusters of metastatic cancer cells and mature DCs, respectively. After washing in PBS, the slides were incubated with diaminobenzidine (Zymed Laboratories, CA) and counterstained with haematoxylin.

The degree of metastasis was grouped into three categories according to keratin immunostaining status: no metastases, immunohistochemical MM (IHC-MM) and histological metastases. IHC-MM was defined as cancer nests <2 mm in size detected immunohistochemically by keratin staining. Mature DC density was defined as the average count of CD83-expressing DCs in ten random fields at a magnification of 400 in a non-cancerous region of the LN medulla. Mature DC density was compared according to the degree of metastasis. LNs that were completely filled with cancer cells were excluded.

**Detection of MM by RT-PCR and quantification of mRNA expression of immunologic parameters in LNs from pathological metastasis-negative cases.** A total of 613 LNs from 25 gastric cancer patients with pathological metastasis-negative LNs were assessed for the presence of MM by amplification of CEA and CK19 mRNA by quantitative RT-PCR (PCR-MM). The presence of PCR-MM was estimated by the relative amounts of CEA or CK19 mRNA standardized by reference internal control gene expression in each sample. The quantification of mRNA levels of the target gene was made using real-time fluorescence detection 'TaqMan™' technology. The amounts of CEA and CK19 mRNA in samples were calculated relative to those in MKN45, and the amounts of CD83, CD86, CD1a and INFγ mRNA in samples were calculated relative to those in non-cancerous lymph nodes. Then the relative quantity of target gene was standardized by that of GAPDH mRNA.

**Statistical analysis.** The statistical significance of intergroup differences in the number of cells expressing CD83 in immunohistological staining was calculated with Kruskal-Wallis and Mann-Whitney U test. The Student's t-test and Mann-Whitney U test were used to analyze the correlation between the expression level of immunologic parameters and the presence of PCR-MM. All p-values were two-sided. A p-value of <0.05 was considered statistically significant.

**Results**

**Distribution and density of mature DCs in the regional LNs in pathological metastasis-positive patients.** A total of 119
LNs from 4 LN metastasis-positive gastric cancer patients were assessed for the presence of cancer cell metastasis and mature DC distribution by haematoxylin and eosin staining and immunohistochemistry. Among 119 LNs from 4 patients, 20 nodes had tumor involvement, as indicated by H&E, as well as tumor cells showing keratin-expression, as indicated by immunohistochemical staining. Moreover, 6 of the histologically negative LNs had occult metastasis, which was indicated only by immunohistochemical staining. The sizes of all 6 keratin-expressing metastatic lesions were <2 mm, and the lesions were considered as IHC-MM. Ninety three LNs had no tumor cells, as indicated by both H&E staining and immunohistochemical staining.

LNs were classified into three groups: 93 nodes without metastasis, 6 nodes with IHC-MM and 20 nodes with pathological metastasis. CD83-positive mature DCs were distributed equally in the LN medulla regardless of the presence of metastatic cancer cells (Fig. 1). We compared the CD83-positive mature DC density among three groups classified according to the degree of metastasis using Kruskal-Wallis analysis of variance. The mature DC density was not different among the three groups (p=0.3208) (Fig. 2).

Detection of PCR-MM and quantification of mRNA expression of immunological parameters in LNs from pathological metastasis-negative cases

Detection of PCR-MM. CEA and CK19 mRNA expression in the 613 lymph nodes from gastric cancer patients ranged from 9.0x10^-2 to 2.7x10^3 and 1.0x10^-2 to 1.7x10^5, respectively. The corresponding values in normal lymph nodes from non-cancerous patients ranged from 0 to 2.0x10^2 and 0 to 1.2x10^3, respectively. In order to exclude false positives, we used the mean values ± 2SD of CEA and CK19 mRNA in normal lymph nodes from non-cancerous patients (1.3x10^3 for CEA and 6.1x10^4 for CK19) as cut-off values. Values above or equal to these cut-off values of CEA and CK19 were defined as CEA and CK19 mRNA positive, respectively. In addition, we defined lymph nodes as PCR-MM positive when either one of CEA mRNA or CK19 mRNA was positive. Using this
cut-off value method, 15 (2.45%) of 613 histologically negative lymph nodes and 6 (24%) of 25 histologically negative cases were determined to be PCR-MM positive (Fig. 3, Table II).

Expression of immunological parameters in PCR-MM-positive LNs. The LNs were separated into two groups based on the presence or absence of PCR-MM as determined by real-time RT-PCR. The expressions of CD83 and CD86 mRNA were significantly higher in PCR-MM-positive than in PCR-MM-negative LNs [2.80 (0-18.7) vs. 1.72 (0-107.1) (p=0.0242), 2.45 (0-22.8) vs. 1.65 (0-75.5) (p=0.0421), respectively]. There was no difference in the expression of CD1a and IFN\(\gamma\) mRNA between the PCR-MM-positive and PCR-MM-negative LNs [0.81 (0-6.7) vs. 0.79 (0-700.0) (p=0.5864), 5.24 (0-819.8) vs. 1.94 (0-376.8) (p=0.1176), respectively] (Table III).

Expression of immunological parameters in LNs from PCR-MM-positive and negative cases. In 151 LNs from 6 PCR-MM-positive cases, 15 LNs were PCR-MM positive and 136 LNs were PCR-MM negative. The expression of immunological parameters in these 151 LNs were compared with 462 LNs from 19 PCR-MM-negative cases. The expression of CD83 and CD86 mRNA was significantly higher in the LNs from PCR-MM-positive cases than in those from PCR-MM-negative cases [2.03 (0-18.7) vs. 1.61 (0-107.1) (p=0.0020), 1.85 (0-29.3) vs. 1.54 (0-75.5) (p=0.0008), respectively]. On the other hand, there was no difference in the expression of CD1a and IFN\(\gamma\) mRNA between the two groups [0.83 (0-617.7) vs. 0.78 (0-700.0) (p=0.3332), 1.84 (0-819.8) vs. 2.03 (0-163.4) (p=0.6941), respectively] (Table IV).

Comparison of immunological parameters among three groups: PCR-MM-positive LNs in PCR-MM-positive cases, PCR-MM-negative LNs in PCR-MM positive cases and PCR-MM-negative LNs in PCR-MM-negative cases. The expression of immunological parameters in LNs were compared among three groups: 15 PCR-MM-positive LNs in 6 PCR-MM-positive cases, 136 PCR-MM-negative LNs in 6 PCR-MM-positive cases and 462 PCR-MM-negative LNs in 19 PCR-MM-negative cases. There were significant differences in the expression of CD83 and CD86 mRNA among the three groups [2.80 (0-18.7) vs. 1.91 (0-12.0) (p=0.0030), 2.45 (0-22.8) vs. 1.80 (0.5-29.3) vs. 1.54 (0-75.5)

Table II. Detection of PCR-MM in LNs from pathological metastasis-negative cases.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of PCR-MM (%)</th>
<th>LN (n=613)</th>
<th>Case (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK19</td>
<td>4 (0.65)</td>
<td>2 (8)</td>
<td></td>
</tr>
<tr>
<td>CEA</td>
<td>11 (1.79)</td>
<td>4 (16)</td>
<td></td>
</tr>
<tr>
<td>CK19 or CEA</td>
<td>15 (2.45)</td>
<td>6 (24)</td>
<td></td>
</tr>
</tbody>
</table>

Values are median (range).

Table III. Expression of immunological parameters in each LN classified by the existence of PCR-MM.

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>PCR-MM(+) LN (n=15)</th>
<th>PCR-MM(-) LN (n=598)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>0.81 (0-6.7)</td>
<td>0.79 (0-700.0)</td>
<td>0.5864</td>
</tr>
<tr>
<td>CD83</td>
<td>2.80 (0-18.7)</td>
<td>1.72 (0-107.1)</td>
<td>0.0242</td>
</tr>
<tr>
<td>CD86</td>
<td>2.45 (0-22.8)</td>
<td>1.65 (0-75.5)</td>
<td>0.0421</td>
</tr>
<tr>
<td>IFN(\gamma)</td>
<td>5.24 (0-819.8)</td>
<td>1.94 (0-376.8)</td>
<td>0.1176</td>
</tr>
</tbody>
</table>

Values are median (range).
There was no difference noted with regard to the expression (values) of CD1a and IFN-γ mRNA \((0.81 (0-6.7) \text{ vs. } 0.81 (0-6.7) \text{ vs. } 0.78 (0-700.0) \text{ (p=0.4381)}, 5.24 (0-819.8) \text{ vs. } 1.78 (0-376.8) \text{ vs. } 2.03 (0-163.4) \text{ (p=0.2030)}, \text{ respectively})\) (Table V, Fig. 4). When comparing the three groups in more detail, the expressions of CD83 and CD86 mRNA were significantly higher in both PCR-MM-positive and negative LNs in PCR-MM-positive cases than in PCR-MM-negative LNs in PCR-MM-negative cases \((CD83: p=0.0191, p=0.0095, \text{ respectively}, \text{ CD86: } p=0.0033, p=0.0311, \text{ respectively})\). However, there was no significant difference in the expressions of CD83 and CD86 mRNA between PCR-MM-positive LNs and PCR-MM-negative LNs in PCR-MM-positive cases \((p=0.0656, p=0.1282, \text{ respectively})\).

### Table IV. Expression of immunological parameters in LNs from PCR-MM-negative and positive cases.

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>PCR-MM(+) case</th>
<th>PCR-MM(-) case</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>0.83 (0-617.7)</td>
<td>0.78 (0-700.0)</td>
<td>0.3332</td>
</tr>
<tr>
<td>CD83</td>
<td>2.03 (0-18.7)</td>
<td>1.61 (0-107.1)</td>
<td>0.0020</td>
</tr>
<tr>
<td>CD86</td>
<td>1.85 (0-29.3)</td>
<td>1.54 (0-75.5)</td>
<td>0.0008</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.84 (0-819.8)</td>
<td>2.03 (0-163.4)</td>
<td>0.6941</td>
</tr>
</tbody>
</table>

Values are median (range). *LN (n=151) from 6 PCR-MM-positive cases were examined. **LN (n=462) from 19 PCR-MM-negative cases were examined.

### Table V. Comparison of immunological parameters among three groups according to the PCR-MM status in LNs.

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>PCR-MM(+) case</th>
<th>PCR-MM(-) case</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=15) LN</td>
<td>(n=136) LN</td>
<td>(n=462) LN</td>
</tr>
<tr>
<td>CD1a</td>
<td>0.81 (0-6.7)</td>
<td>0.81 (0-6.7)</td>
<td>0.78 (0-700.0)</td>
</tr>
<tr>
<td>CD83</td>
<td>2.80 (0-18.7)</td>
<td>1.91 (0-12.0)</td>
<td>1.61 (0-107.1)</td>
</tr>
<tr>
<td>CD86</td>
<td>2.45 (0-22.8)</td>
<td>1.80 (0.5-29.3)</td>
<td>1.54 (0-75.5)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5.24 (0-819.8)</td>
<td>1.78 (0-376.8)</td>
<td>2.03 (0-163.4)</td>
</tr>
</tbody>
</table>

Values are median (range). *LN (n=151) from 6 PCR-MM-positive cases were classified according to the existence of PCR-MM. **LN (n=462) from 19 PCR-MM-negative cases were examined.

Figure 4. CD83 and CD86 mRNA expression in regional LNs according to the PCR-MM status. CD83 and CD86 mRNAs were quantified by amplifying mRNA by quantitative RT-PCR. The amounts of target mRNA in samples were calculated relative to those in normal lymph nodes from non-cancer patients and standardized according to those of GAPDH mRNA.
Discussion

In this study, we examined the immunological response including the maturation status of DCs in regional lymph nodes of gastric cancer patients. In metastasis-positive cases, an immunohistochemical study showed that the distribution and density of CD83-positive mature DCs were independent of the extent of metastasis in lymph nodes. In metastasis-negative cases, real-time RT-PCR showed that the expression levels of CD83 and CD86 mRNA were significantly higher in PCR-MM-positive lymph nodes compared with PCR-MM-negative lymph nodes. Comparable results were obtained in comparing PCR-MM-positive cases with negative cases. These results indicate that the immunological response in regional lymph nodes, including the maturation of DCs, is activated when PCR-MM occurs. In addition, further maturation of DCs does not occur after the pathological metastasis is established. This immune response is not restricted to the lymph nodes in which PCR-MM is being developed, but takes place in all of the regional lymph nodes.

The TNM classification stipulates that MM are metastases that measure ≤2 mm but >0.2 mm. First, we examined the immunological response by investigating the maturation status of the DCs histologically in metastasis-positive cases. We found that the distribution and density of mature DCs in LNs were independent of metastasis status in pathological metastasis-positive patients. It was assumed that DCs had been activated in an earlier stage of metastasis than the stage of histologically detectable MM. Therefore, we examined the immunological response by RT-PCR at the stage before pathological MM was established. RT-PCR is a highly sensitive molecular technique to detect the minute epithelial mRNA of cancer cells that could not be detected by immunohistochemical staining. In the present study, we defined the MM detected only by RT-PCR as PCR-MM and that detected by IHC as IHC-MM. We distinguished PCR-MM as the initial state of MM, in contrast to IHC-MM in which metastatic foci was <0.2 mm but could be detected histologically. The relationship between the histological metastasis in lymph nodes and the immune response was reported in previous studies. Focusing on gastric cancer, Tsujitani et al. (32) investigated the infiltration of DCs into regional lymph nodes by using immunohistochemical staining against S-100 protein. They found that there was a significant correlation between the extent of infiltration by DCs in the primary tumor and that of the peritumoral nodes. They also established that when the primary nodes were involved, the extent of infiltration by DCs was elevated in both the primary and secondary nodes. These authors concluded that DCs in the regional lymph nodes can set up immunological defense mechanisms against lymph node metastasis when metastasis is absent or limited to the primary nodes. Our data demonstrated that the distribution and density of mature DCs in IHC-MM-positive LNs were not different from those in histological metastasis-negative and positive LNs. This observation suggests that the immune response may show a tendency toward immunotolerance or immunosuppression and that the defensive mechanism against cancer may decrease when the histological metastasis, including IHC-MM, is established. The different results in the two studies may result from the different surface markers used to detect the mature DCs. Tsujitani et al. used S-100 protein as a marker of DCs. However, S-100 protein is expressed in several types of DCs, including immature and mature DCs. In contrast, most of the recent immunological studies, including the present study, used CD83 as a marker of mature DCs. Moreover, the possibility that the primary tumor or its metastasis may cause immune suppression is shown from the examination of several cytokine profiles and the function of DCs (35). Therefore, we propose the hypothesis that the immunological activation, based on DC maturation, had already occurred at a stage before a histological metastasis was detected, and that immune suppression was induced as a result. As far as we know, no information is available on DC maturation accompanied by PCR-MM in the regional lymph nodes of cancer cases. To investigate the relationship between metastasis and the immune response at the earlier stage, we examined the relation between PCR-MM and the maturation status of DCs. The term, ‘PCR-MM-positive’ means the existence of minute epithelial mRNA. It is uncertain whether or not the cancer cells metastasize to the lymph nodes at the stage of PCR-MM. Because DCs capture tumor-specific antigens in the primary tumor, process the antigens into peptides and migrate to the regional lymph nodes, the possibility that the fragments of mRNA processed and presented by DCs were amplified and recognized as ‘PCR-MM positive’ cannot be excluded. In any case, we established that the immune response caused by the maturation of the DCs was initiated when small amounts of tumor-specific antigens or cancer cells were admitted into the regional lymph nodes, and that the activation of the immune response was almost ending at the stage when the metastasis in the lymph nodes was observed as IHC-MM. It is uncertain whether the PCR-MM can grow to be recognized as histological metastasis or is excluded by the subsequent immune response. In addition, it is still undetermined whether the DC maturation is triggered after establishment of PCR-MM or whether the immune response is already activated by the signal from the primary tumor before the entry of PCR-MM into the lymph nodes. Di Girolamo et al. (36) investigated the immune response by immunohistochemistry on CD4+ T cells, CD8+ T cells, B lymphocytes, DCs and NK cells in metastasis-free regional lymph nodes draining different human epithelial tumors and compared the results with those of control lymph nodes from patients with no malignancy. The results indicated that regional lymph nodes contained reduced numbers of almost all immune cells, except CD57+ cells, and the authors concluded that the decrease of several immunocytes might be related to a deficient anti-tumor immune response and subsequent tumor progression in patients with cancer. Laguens et al. (37) also demonstrated that lymph nodes draining cancer sites had significantly fewer CD1a+ DCs and CD86+ DCs than did lymph nodes from patients without malignancy. In addition, Kierscher et al. (35) showed that tumors can produce a number of immunosuppressive factors that block DC maturation and induce early apoptosis of monocyte-derived DCs. These previous reports indicated that immunosuppression took place in the regional lymph nodes draining cancer sites. In the present study, we found that the maturation of DCs in regional lymph nodes is activated when PCR-MM is present. The tumor-specific immune response might be
triggered by the generation of metastasis to regional lymph nodes, but only at the PCR-MM stage. Further activation of DCs and subsequent immunological response in the lymph nodes might decrease because immunosuppressive signals are produced by the primary cancer at the stage after IHC-MM formation.

In order for naïve T-cells to acquire the functions of effector cells, not only tumor-specific antigens associated with MHC molecules but also a second signal provided by co-stimulatory molecules, which are expressed on T-cells, are needed (14). Two co-stimulatory molecules designated CD80 and CD86 have been identified on professional APC, including DCs (38). CD80 and CD86 share two receptors on T cells, which have been designated CD28 and CTLA-4, respectively (39,40). When either CD80 or CD86 binds to the CD28 receptor, a co-stimulatory signal is produced that results in a Th1 immune response. In contrast, binding of either CD80 or CD86 to CTLA-4 appears to activate a Th2 immune response, which results in T-cell anergy (41,42). We demonstrated that CD83 and CD86 mRNA expression is elevated in PCR-MM-positive LNs and patients. These results suggest that when PCR-MM occurs the DCs become activated and mature, and they are then able to present antigens to T-cells. However, there was no difference between the expression levels of IFNγ in PCR-MM-positive LNs and patients. Their suggestion that the immune response might be stimulated by submicroscopic tumor cells secreted does not transmit to naïve T-cells although the DCs secreted by naïve T-cells, but it is possible that the signal to secrete does not transmit to naïve T-cells although the DCs have already been activated. Another possibility might be that the Th1 response is controlled by binding of CD86 to the CTLA-4 receptor instead of the CD28 receptor. CTLA-4 behaves as a negative regulator of the proliferation and the effector function of T-cells.

Recently, the immune status in the lymph nodes, especially in sentinel lymph nodes (SLNs), was investigated in several malignancies. The immune status of SLNs seems to be suppressed in the absence of cancer cells, although this idea is still controversial (43). Matsuura et al (44) investigated the antigen-specific immunity of SLNs based on the maturation of DCs, Th1 and Th2 responses, and T-reg cell responses in lymph node-negative breast carcinoma patients. By using flow cytometry and RT-PCR analysis they observed that the immunological status of SLNs, including DC maturation and Th1 responses, is depressed in SLNs before metastasis. They showed that patients with no metastasis had an increased secretion of cytokines in the SLNs, whereas patients harboring lymph-node MM showed no increase of cytokine secretion in the SLNs. This finding suggested that the lymphocytes in tumor-free SLNs could have been stimulated by a very small number of tumor cells that were not detectable by haematoxylin and eosin or immunohistochemical staining. Thus, it seems that a large immune response can be induced in SLNs that are melanoma-free, and that this response might be stimulated by submicroscopic tumor cells or soluble tumor antigens, resulting in the production of cytokines (43). Their suggestion that the immune response might be stimulated in the initial stage of histological or immunohistochemical metastasis development appears to be consistent with our data. Sentinel node biopsy in early gastric cancer is now widely used to predict nodal status. In our institution, lymphatic basin dissection with limited gastrectomy is performed in early-stage gastric carcinoma patients, especially if there is no metastasis detected in SLNs on frozen sections. Lymphatic basins are defined as the lymphatic zones divided by the stream of the stained lymphatic canals. Because it is difficult to identify the SLNs with certainty, we regard the lymphatic basin as the most important lymphatic area in which lymph node metastasis first develops (33,46). We have confirmed the progression pattern of lymph node metastasis: nodal metastasis occurred first in SLNs, then spread to non-SLNs within the lymphatic basin, and rarely extended outside the lymphatic basin. In this study, we also compared the immune response pattern in SLNs with that of non-SLNs. The DC maturation status in SLNs was no different from that in non-SLNs (data not shown). On the other hand, an activated immune response is not restricted to the lymph nodes in which PCR-MM is developing, but takes place in all regional lymph nodes within the lymphatic basin. This suggests that when PCR-MM occurs the immune response develops in all lymph nodes in the same lymphatic basin, regardless of whether the lymph node is an SLN or not.

In summary, we conclude that in gastric cancer the activation of the immune response by maturation of DCs takes place at the stage before metastasis has occurred, especially the stage of MM, and that it develops in the regional lymph nodes. Our results might provide a step toward clarifying the self-defense mechanism against lymph node metastasis in carcinoma. Further studies are needed to clarify whether immunological defense mechanisms work effectively to prevent lymph node metastasis.

References


