Background: To clarify the pathophysiological role of tumor-associated macrophages (TAMs), we performed clinicopathological analysis of CD68+ cells in 70 cases of human endometrial cancer. Materials and Methods: Using immunohistochemistry for CD68, we classified CD68+ cells into four groups: (a) those infiltrated into cancer cell nests or in close contact with cancer cells (nest TAM); (b) those in necrosis in the tumor center (hot-spot TAM); (c) those infiltrated into cancer stroma (stroma TAM); and (d) those distributed along the invasive margin of a tumor (Margin TAM). Results: The aggregation of nest TAM related to high relapse-free survival rate after surgery. On the contrary, increased hot-spot TAM was a hazard to relapse-free survival and was proportionately-associated with clinical stage, myometrial invasion and histological differentiation. The extent of stroma TAM was associated with the presence of lymph node metastasis. Conclusion: Our findings demonstrate that the histological location of infiltrated TAMs may be taken into account in the clinical evaluation of endometrial cancer.

Although many macrophages – tumor-associated macrophages (TAMs) – infiltrate into tumor tissues, it remains unclear how macrophages affect tumor inhibition or progression. Infiltration of TAMs has long been considered to indicate host-immune reaction (1-3). Recently, however, several studies have reported that TAMs play a key role in tumor angiogenesis, which ultimately modulates tumor growth and invasion (4, 5). Consequently, the results obtained from clinical studies so far are controversial (6).

Malignant solid tumors usually develop into compact masses with intervening stromal tissues and are often associated with areas of necrotic foci, as well as a peripheral vital zone of viable tissue. Various microenvironments are present in malignant tumor tissues: low oxygen tension, subsequent tissue necrosis or angiogenesis, focal inflammation, immune response to cancer and degradation and remodeling of extracellular matrix (ECM). Moreover, recent investigations have provided clear evidence that TAMs actively participate in each process during tumor progression (7, 8). We hypothesised that the histological location of infiltrated macrophages helps explain the ambivalent role of TAMs in solid tumor (9).

To date, only a few studies have evaluated the role of TAMs from the standpoint of their spatial distribution in cancer tissue: cancer nests, necrotic area, stroma and tumor margin (Figure 1). Leek et al. (10) elucidated that TAMs along the invasive tumor margin or near necrotic foci, involved with hypoxic areas in breast cancer, produced angiogenic factors, and that increased TAMs correlated with angiogenesis and poor prognosis. On the contrary, Shimura et al. (11) pointed out that CD68+ cells in contact with cancer cells produced a high density of cytotoxic mediators [e.g. tumor necrosis factor (TNF)-α and inducible NO synthase (iNOS)] in prostate cancer. More recently, we have shown that aggregation of TAMs within cancer cell nests without a necrotic area in gastric cancer has a beneficial effect on the host in terms of tumor-cell apoptosis induction and cytotoxic T lymphocytes infiltration. Although there are a few reports on the role of TAMs in endometrial cancer (12, 13), the precise correlation of TAM density in each histological
location with clinicopathological features and prognosis is still obscure. To address these questions, we evaluated the distribution of CD68-positive cells on the basis of histological location in 70 consecutive patients with endometrial cancer.

**Patients and Methods**

**Patients.** This study included 70 primary endometrial carcinoma patients who were consecutively admitted, treated and followed-up at the Department of Obstetrics and Gynecology, Kanazawa University Hospital, Japan, from January 1995 to December 2002. Endometrial cancers were classified according to the International Federation of Gynecology and Obstetrics (FIGO) surgical staging system (1988) as follows: stage IA, n = 12; stage IB, n = 32; stage IC, n = 8; stage II A, n = 3; stage II B, n = 3; stage III A, n = 4; stage III B, n = 6; and stage IV B, n = 2. Tumors were classified histologically according to the Histopathology-Degree of Differentiation (FIGO 1988) as follows: Grade 1, n = 38; Grade 2, n = 13; and Grade 3, n = 19. Because the depth of myometrial invasion is associated with other prognostic factors such as the grade of the tumor, primary tumors were classified as follows: (a) tumor limited to endometrium, n = 17; (b) invasion to ≤ 1/2 myometrium, n = 37; and (c) invasion to > 1/2 myometrium, n = 16. All patients underwent a total abdominal or radical hysterectomy plus bilateral salpingo-oophorectomy. At the time of celiotomy, peritoneal fluid samples were obtained for cytology testing. Systemic pelvic lymphadenectomy was performed in 51 (72.9%) patients. Paraortic lymph node sampling was performed in 2 patients because of visible or palpable enlarged lymph nodes. No patients had remaining macroscopic tumors or known distant metastasis at the time of surgery. High-risk patients (e.g., deep myometrial invasion, cervical involvement, special histology, positive for peritoneal cytology) underwent external radiotherapy and/or six cycles of TJ chemotherapy [Paclitaxel: 180 mg/m², Carboplatin: according to Chatelut’s formula (AUC=5 mg·min/ml)] as postoperative adjuvant therapy. Patient treatment was followed with a gynecological examination, recording of laboratory data, transvaginal/abdominopelvic ultrasonography and a radiological investigation. Data from regular follow-up visits to the outpatient department were stored in a database specially designed for endometrial carcinoma patients. A telephone inquiry to update the present status of all surviving patients was made in July 2003. The exact date of disease recurrence was collected from the referring physician or from the physician who attended the patient for the initial diagnosis of the recurrence. All treatments and clinical research were conducted with informed consent.

**Immunohistochemistry.** Representative paraffin sections containing both the normal endometrium and the invasive front of the tumor tissue were selected for immunohistochemical staining. Slides were deparaffinized, rehydrated in graded alcohols and placed in PBS solution. Epitope retrieval was done by enzymatic digestion with trypsin for 30 minutes at 37°C (TRYPSIN Tablets, SIGMA-ALDRICH CHEMIE Gmbh, Steinheim, Germany). Endogenous peroxidase activity was quenched by dipping in 3% H₂O₂ for 30 minutes, and non-specific binding was blocked by treating slides with normal rabbit serum for 30 minutes. Monoclonal mouse anti-human CD68 antibody (Clone PG-M1, Mouse IgG3k; DAKO A/S, Glostrup, Denmark; 1:50 dilution) was used at room temperature for 2 hours incubation. The streptavidin-biotin peroxidase complex (SABC) method was used for the immunohistochemical steps which were done with a commercially available kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan), as previously described (14). Color development was done with peroxidase substrate 3-amin-9-ethylcarbazole. Human tonsil tissue was used for a positive control. The primary antibody was replaced by non-immune serum in the negative control slide.

**Evaluation of tumor-associated macrophages.** Using immunohistochemistry for CD68, we classified CD68+ cells into four groups: (a) those infiltrated into cancer cell nests or in close contact with cancer cells; (b) those in necrosis in the tumor center; (c) those infiltrated into cancer stroma; and (d) those distributed along the invasive margin of a tumor (Figure 1). For counting TAMs, the sections were first evaluated at x100 magnification under a light microscope, and five representative areas where TAMs accumulated at greater density were identified. TAMs were then counted at x400 magnification and the average of the five values was used for statistical analysis.

**Statistical analysis.** The Mann-Whitney U-test was used to analyze the distribution of CD68+ cells according to several clinicopathological variables. Relapse-free survival rates were calculated from the date of surgery to the date of disease recurrence or the date of the end point. In the analysis of relapse-free survival rates, those who died of causes unrelated to endometrial cancer and those who had no detected evidence of disease recurrence were considered relapse-free. Life tables were computed using the Kaplan-Meier method while the log rank test was used to assess statistical significance. Cox proportional hazard analysis was used to determine the relative contribution of various factors to the risk of recurrence. A p value of < 0.05 was considered to indicate statistical significance. All statistical analyses were performed using the statistical package StatView version 5.0 for Macintosh computer (Abacus Concepts, Inc., Berkeley, CA, USA).

**Results**

**Characteristics of the patients.** The average age of the patients at the time of surgery was 57.3 years old (range, 26-78). Patients with endometrial cancer included: 22 with premenopausal status, 4 with perimenopausal status and 44 with postmenopausal status. The mean of preoperative body mass index (BMI) of the patients was 24.0 (range, 16.9 - 32.9). Among the 70 patients, 12 patients (17.1%) had relapses of endometrial cancer at the time of the last follow-up. The median follow-up time for all patients was 3.28 years (range, 0.15 - 8.50 years).

**CD68 expression at different localizations.** Most of the CD68+ cells were distributed along the invasive margin of tumor, in the necrotic foci and tumor stroma. CD68+ cells were also detected within the cancer cell nest (Figure 2a-e). When normality was evaluated by normal distribution plots and histograms for variables, the number of TAM showed some degree of being positively skewed. Therefore, the results in the text and tables are given as median and interquartile range (IQR) unless otherwise stated. The median value (IQR, maximum - minimum) of TAM in each
Localization was as follows: nest TAM, 10.7 (15.8, 0.8 - 50.4); hot-spot TAM, 12.1 (35.0, 0.0 - 111.8); stroma TAM, 19.1 (20.4, 0.6 - 61.6); and margin TAM, 19.3 (22.4, 0.0 - 130.0).

There was little correlation among the number of TAM at each locality (Spearman rank correlation coefficients in all comparison: less than 0.40).

In clinicopathological analysis, increased hot-spot TAM positively-correlated with disease progression. There was an association between stroma TAM and lymph node metastasis, however, no significant relationship between nest or margin TAM and the clinical parameters was found (Tables I-IV).

Survival analysis. The median values of nest TAM, hot-spot TAM, stroma TAM and margin TAM were used for the cut-off point to stratify patients into 2 groups per case. Analysis of relapse-free survival (Figure 3a-d) showed that low nest TAM and high hot-spot TAM were the risk factors negatively influencing the relapse-free survival rate. Margin TAM and stroma TAM had no statistically significant impact on relapse-free survival.

After adjusting for several prognostic factors (age, FIGO stage, myometrial invasion, histology, hot-spot TAM, nest TAM), a trend toward an association between the degree of nest TAM infiltration and risk of recurrence ($p=0.0729$, 95% CI: 0.859-30.684) was found as assessed by Cox proportional hazard analysis.

Discussion

Three main points emerged from this study: 1) The aggregation of CD68+ cells infiltrated within cancer cell nests or in close contact with cancer cells (nest TAMs) had a beneficial effect on the relapse-free survival of patients with endometrial cancer. 2) Increased CD68+ cells in a tumor center necrosis (hot-spot TAMs) were a hazard to relapse-free survival for endometrial cancer patients, and were proportionately associated with clinical stage, myometrial invasion and histological differentiation. 3) The extent of CD68+ cell infiltration within cancer stroma (stroma TAM) was associated with the presence of lymph node metastasis.
Figure 2a-e. Immunohistochemical staining of endometrial cancer with anti-CD68 antibodies (original magnification: a, x40; b, x200; c, x200; d, x200; e, x200). (a) Representative sections of invasive margin of endometrial cancer with central necrosis are shown. (b) CD68+ cells within cancer cell nests (Nest TAM). (c) CD68+ cells in necrotic areas with large neutrophil clusters (Hot spot TAM). (d) CD68+ cells infiltrated in cancer stroma (Stroma TAM). (e) CD68+ cells along the invasive margin of tumor (Margin TAM).
When appropriately activated, macrophages can trigger a tumor-destructive reaction and kill cancer cells. The immunohistochemical detection of TNF-α and iNOS produced by infiltrating macrophages has been confirmed in breast (15, 16), ovarian (17) and prostate (11) cancer. These tumor-killing soluble factors (e.g., TNF-α and NO) and other cytotoxic effectors act in a paracrine manner and stimulate cytotoxicity of TAM itself in an autocrine or paracrine manner (2, 18). This implicates that the cytotoxicity of activated macrophages is exerted through cell-to-cell contact or infiltration into the cancer nest in solid tumor tissue. In fact, Shimura et al. (11) revealed that cytotoxic factors by TAMs in prostate cancer differed between TAMs residing within the tumor nests and those located outside the tumor nests. Moreover, the aggregation of TAMs was an independent predictor for improved disease-free survival after surgery. We also have disclosed the functional significance of TAMs infiltrated into cancer cell nests in the tumor-immune system of advanced gastric cancer (14). In the present study, we found that the aggregation of nest TAMs has a beneficial effect leading to good prognosis. Thus, we suggest that nest TAM is cytotoxic enough to kill cancer cells.

On the contrary, increased hot-spot TAMs were associated with poor prognosis, as well as other prognostic factors such as clinical stage, myometrial invasion and histological differentiation. Leek et al. (9) showed a strong relationship between increased TAM counts, reduced relapse-free survival and reduced overall survival in breast cancer patients. Additionally, Leek et al. (19) revealed that focal TAM infiltration was correlated with the degree of tumor necrosis in invasive breast carcinoma and that necrosis characterized tumors possessing an aggressive phenotype, such as high tumor grade, larger size and low estrogen-receptor status. Therefore, aggressive tumors rapidly outgrow their vascular supply in certain areas, leading to areas of prolonged hypoxia within the tumor and, subsequently, to necrosis. This, in turn, may attract a TAM into the tumor, which then contributes to the angiogenic process, giving rise to associations between extensive necrosis and aggregation of TAM and enriched angiogenesis. It is well established that extensive neovascularization as well as massive TAM infiltration in an area of central necrosis in tumor is a poor prognostic factor for several forms of human cancers (20-22). These data indicate that hot-spot TAMs may play a central role in tumor angiogenesis and tumor progression, and that assessment of hot-spot TAM counts can be used clinically to predict an outcome in various types of cancers (12, 23, 24).

Central necrosis is often present in solid malignant tumor and is accompanied by an aggregation of TAMs. These necrotic areas associate with low-oxygen tension, which

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IQR*: interquartile range
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Central necrosis is often present in solid malignant tumor and is accompanied by an aggregation of TAMs. These necrotic areas associate with low-oxygen tension, which
stimulates the promoter [e.g., hypoxia-inducible factor (HIF)] of the infiltrating macrophages and lets them produce angiogenic factors such as VEGF, TNF-α, and TGF-β (15, 25). Griffiths et al. (26) revealed that, when a multicellular tumor spheroid was co-cultured with human macrophages, the macrophages accumulated rapidly in the hypoxic area of these three-dimensional cultures of tumor cells. Further, Griffiths demonstrated the potential of using these macrophages as a cell-based delivery system for gene-dependent enzyme prodrug therapy. Genetically-engineered macrophages infiltrated a tumor spheroid, up-regulated the therapeutic hypoxia-response elements (HRE)-containing transgenes in a hypoxia-dependent manner, and significantly killed tumor cells in the presence of the prodrug cyclophosphamide by activating P4502B6. These findings suggest that macrophages may be used to deliver HRE-regulated therapeutic genes specifically to hypoxic tumor areas. However, the mechanism attracting TAMs to hypoxic areas with tumor necrosis is still obscure. Recent investigations point to the following three hypotheses: 1. TAMs recruit the scavenging of apoptotic bodies and necrotic cell debris in these areas. 2. TAMs migrate randomly around the tumor site and subsequently adjust themselves to the hypoxic tumor environment for survival under hypoxic conditions. 3. TAMs are immobilized in hypoxic regions by the inhibitory effect of hypoxia on TAM migration induced by monocyte chemoattractant protein (MCP) -1 and macrophage inflammatory protein (MIP) -1α. Further studies are needed to clarify this point.

There is no clear consensus regarding the functional significance of TAM infiltrated into cancer stroma or TAM distributed along the invasive margin of a tumor. Ohtani et al. (27) theorized that CD68+ cells along the invasive margin of colon cancer may be involved in an immune reaction in association with T lymphocytes present at the same location to suppress tumor spread. Migita et al. (28) reported on the in situ expression of the matrix-degrading enzyme activity of macrophages along the tumor margin in gastric carcinoma and discussed its possible pathophysiological significance. Meanwhile, angiogenic factors and their promoter (e.g., HIF) were detected in CD68+ cells in cancer stroma or along the invasive tumor margin in many varieties of tumors (29, 30). Our results established that stroma and margin TAM did not contribute to the capacity to predict the time to recurrence of endometrial cancer and that stroma TAM was associated with the presence of lymph node metastasis. Further studies are needed to clarify the role of stroma/margin TAM in human solid tumor.

Although human solid tumors involve the infiltration of many TAMs as host immune or inflammatory cells (31, 32), the cancer cells cause recurrence through evading the host
immune surveillance in many ways and continue to grow. Recently, Pollard (33) suggested that the tumor microenvironment educated the infiltrated macrophages to lend a helping hand to tumor progression and the metastasis process (angiogenesis, matrix breakdown and tumor cell motility). Our findings revealed that nest TAM had significant predictive value for determining endometrial carcinoma recurrence after surgery. We suppose that strategies aimed at increasing the infiltration of re-educated-nest TAM or transforming the tumorigenic microenvironment into a tumor-suppressive one may prove useful adjuvants to immune therapy in endometrial cancer patients.

In conclusion, our findings demonstrate that the histological location of infiltrated TAMs may be taken into account in the clinical evaluation of endometrial cancer. These results have confirmed the findings of other investigators and extended them in several important directions.

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**References**


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