Angiogenesis is indispensable to tumor development and proliferation. The aim of this study was to investigate whether the expression of monocyte chemotactic protein-1 (MCP-1) and of thymidine phosphorylase (TP) correlates with the angiogenesis and clinicopathologic features in cardiac myxoma. Paraffin-embedded specimens of 17 resected cardiac myxomas were immunohistochemically stained for MCP-1, CC chemokine receptor-2 (CCR-2), TP, CD31, and CD68. Correlations among MCP-1 expression, TP expression, microvessel count (determined by CD31 staining), macrophage count (determined by CD68 staining), and the clinicopathologic features of the patients were analyzed statistically. Immunohistochemical analysis revealed that MCP-1 and TP were expressed in myxoma cells, as well as in stromal cells such as infiltrating cells, fibroblast-like cells and endothelial cells. CCR-2 was abundantly expressed in stromal infiltrating cells in all myxomas and occasionally in the endothelial cells. In the tumor stroma, the major source of MCP-1, TP and CCR-2 was macrophages, and the sites of positive staining for MCP-1, TP and CCR-2 matched in most of the myxomas. Statistical analysis revealed that the proportions of MCP-1-positive myxoma and stromal cells, and TP-positive myxoma and stromal cells significantly correlated with increased microvessel count. The proportions of MCP-1-positive myxoma and stromal cells significantly correlated with the proportion of TP-positive stromal cells. The mean microvessel count in myxomas with both high tumor and high stromal MCP-1 or TP expression was significantly higher than that in myxomas with low tumor and low stromal MCP-1 or TP expression. Small tumors (≤55 mm in diameter) exhibited high MCP-1 or TP expression, and the microvessel count in small tumors was significantly higher than in large myxomas. Although the difference was not significant, myxomas with both high tumor and high stromal MCP-1 expression had a higher macrophage count than other myxomas. These results indicate that in cardiac myxoma, MCP-1 and TP may be regarded as important angiogenic signals accompanying growth. (Circ J 2003; 67: 54–60)

Key Words: Angiogenesis; Cardiac myxoma; CC chemokine receptor-2; Monocyte chemotactic protein-1; Thymidine phosphorylase

Angiogenesis, which is a complex biological process regulated by a number of cytokines or growth factors secreted by tumor and/or stromal cells, is indispensable to tumor development and proliferation! Monocyte chemotactic protein-1 (MCP-1) is a potent monocyte chemotactrant involved in various pathological conditions2–6 and several lines of evidence suggest that MCP-1 is up-regulated in many tumor types7–9 and contributing to angiogenesis. MCP-1 can induce intra-tumor infiltration of macrophages, which may subsequently induce tumor angiogenesis through production of angiogenic factors such as basic fibroblast growth factor, vascular endothelial growth factor (VEGF), thymidine phosphorylase (TP), tumor necrosis factor-α, and interleukin (IL)-810–16. In addition, MCP-1 may also have a direct effect on tumor angiogenesis by inducing chemotaxis of endothelial cells, as evidenced by the detection of CC chemokine receptor-2 (CCR-2), a receptor for MCP-1, in these cells17. These findings indicate that MCP-1 contributes to tumor angiogenesis via macrophage attraction and/or via direct chemotactic stimulation of endothelial cells.

On the other hand, TP is an angiogenic enzyme shown to be identical to platelet-derived endothelial cell growth factor18. The precise mechanism of its angiogenic action is unknown, but 2-deoxy-D-ribose, a product from thymidine through the catalytic action of TP, has been shown to have chemotactic activity in vitro and angiogenic activity in vivo19,20. The concentration of TP has been found to be elevated in tumor tissues compared with adjacent normal tissues, and reported to correlate with microvessel density and a poor clinical prognosis in patients with various tumor types, including breast, bladder, esophagus, and gastrointestinal carcinomas21–26. Cardiac myxoma is the most common primary tumor of the heart in adults. In a previous study, we showed that VEGF expression is associated with angiogenesis, which was evaluated by microvessel count in cardiac myxoma27. In the present study, to further understand the nature of the angiogenesis of cardiac myxoma, we examined MCP-1 and TP expression by immunohistochemistry, and investigated their roles in tumor angiogenesis and tumor growth.
Methods

Patients and Specimens
Tissue samples of 17 patients with cardiac myxoma who underwent cardiac surgery at the Second Department of Surgery, Shinshu University Hospital, and affiliated hospitals between 1988 and 2000 were studied. The samples included those of 13 cases used in our previous study. The tissues were fixed in 10% formalin buffered with phosphate at pH 7.4 and embedded in paraffin. Serial sections, 4 μm thick, were cut from paraffin blocks, mounted on glass slides and examined by immunohistochemistry. Two tissue samples of the atrial septum and 2 from the atrium were examined as controls.

Immunohistochemistry
The paraffin sections were stained for MCP-1, CCR-2, TP, CD31, and CD68 by the avidin-biotin-peroxidase complex method. Briefly, the sections were deparaffinized, rehydrated, and then boiled in 0.01 mol/L citrate buffer (pH=6.0) for 15 min in a microwave oven. Endogenous peroxidase was suppressed by treatment with 0.3% hydrogen peroxide in methanol for 30 min, and the sections were incubated with normal animal serum for 30 min to reduce nonspecific binding. Sections were then incubated with primary antibodies for 120 min. Monoclonal antibodies used in this study were: anti-human MCP-1 antibody (5D3-F7, diluted 1:50; Pharmingen, CA, USA), anti-human CCR-2 antibody (48607.121, diluted 1:100; Genzyme/Technne, MN, USA), anti-human TP antibody (654-1, diluted 1:100; Nippon Roche Research Center, Kanagawa, Japan), anti-human CD31 antibody (JC70A, diluted 1:100; Dako A/S, Glostrup, Denmark), and anti-human CD68 antibody (KP1, diluted 1:100; Dako A/S). Sections were incubated with biotinylabeled second antibody diluted 1:200 for 60 min, then with avidin-biotin-peroxidase complex diluted 1:100 (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 60 min. The immunoreaction was visualized by developing sections in 0.025% diaminobezidine and 0.05% hydrogen peroxide, followed by counter-staining with hematoxylin. For negative controls, non-immunized mouse immunoglobulin-G was substituted for primary antibody at the same concentration as the test antibody in every run.

Evaluation of Immunostaining
The immunoreactivity of both MCP-1 and TP was demonstrated in the cytoplasm of both myxoma and stromal cells, so the staining results were evaluated separately. For the myxoma cells, the proportions of MCP-1- or TP-positive cells (0–100%) were calculated in the three most highly stained areas on each slide at 200-fold magnification, and the mean percentage of cells stained was regarded as the tumor expression of MCP-1 or TP. In each field, 9–210 myxoma cells were examined. MCP-1 expression varied from 13.6% to 72.75% with a mean value of 46.02%, and TP expression varied from 0% to 98% with a mean value of 37.59%. Myxomas were considered to show...
high tumor MCP-1 or TP expression when 50% or more of the cells were stained positively, whereas myxomas with less than 50% staining were considered to show low tumor expression.

In the tumor stroma, the expression of MCP-1 or TP was evaluated following the same procedure used for the myxoma cells. In each field, 31–338 stromal cells were examined. Because MCP-1 expression varied from 22.13% to 76.82% with a mean value of 45.79% and TP expression varied from 17.65% to 94.63% with a mean value of 48.89%, we again selected 50% as the cutoff point.

Microvessel Count and Macrophage Count

Microvessels that stained for CD31 antigen were counted in the 5 areas of highest vascular density at 200-fold magnification of the light microscope. The microvessel count was expressed as the mean number of microvessels in each section, as shown in a previous report.28

Macrophages were stained by anti-CD68 antibody, and the positively stained cells were counted in the 5 areas of most accumulation at 200-fold magnification of the light microscope. The macrophage count was expressed as the mean number of macrophages in each section.

Statistical Analysis

Correlations among MCP-1 expression, TP expression, microvessel count, and CD68 positive cells were evaluated by Pearson’s correlation analysis. Correlation between clinicopathologic features and MCP-1 or TP expression was analyzed using Fisher’s exact test. Significant differences in microvessel count and macrophage count were analyzed by the Mann-Whitney U test. Differences were considered significant when the p value was less than 0.05.

Results

Immunostaining for MCP-1 and CCR-2

Immunoreactivity for MCP-1 was found in the cytoplasm of tumor cells in all myxomas (Fig 1A) and heterogeneity was found in the distribution of MCP-1-positive tumor cells in most myxomas. Of the 17 myxomas, 7 (41.2%) showed high tumor MCP-1 expression.

In the tumor stroma, MCP-1 was abundantly expressed in infiltrating cells in all myxomas, and occasionally in the endothelial cells of a few blood vessels in 3 (17.6%) of the 17 myxomas. Of the 17 myxomas, 5 (29.4%) had high stromal MCP-1 expression.

CCR-2 was abundantly expressed in stromal infiltrating cells in all myxomas, and occasionally in the endothelial cells of a few blood vessels in 3 (17.6%) of the 17 myxo-
mas (Fig 1B). The infiltrating cells expressing CCR-2 were mainly macrophages, and sites of CCR-2 staining were similar to those for MCP-1 (Fig 2A–C).

In the control tissues, positive immunoreactivity for either MCP-1 or CCR-2 was not observed.

**Immunostaining for TP**

TP immunoreactivity was found in the tumor cells in 12 (70.6%) of the 17 myxomas (Fig 1C). The staining was cytoplasmic and the distribution of TP-positive myxoma cells was heterogeneous. Of the 17 myxomas, 6 (35.3%) showed high tumor TP expression.

In the tumor stroma, TP expression was found in infiltrating cells, fibroblast-like cells, and endothelial cells. The majority of TP was the infiltrating cells and the areas that were highly stained for TP usually had a large number of blood vessels (Fig 2D). Because similar staining patterns were observed for TP, CCR-2, and CD68 in most cases, we presume the majority of TP-positive cells were infiltrating macrophages that were also CCR-2 positive (Fig 2B–D). Of the 17 myxomas, 6 (35.3%) showed high stromal TP expression.

The control tissues were not immunoreactive for TP.

**Immunostaining for CD31 and CD68**

Microvessels were detected using anti-CD31 antibody

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**Table 1** Correlation Between Extent of MCP-1 Expression and Clinicopathologic Features of Cardiac Myxoma

<table>
<thead>
<tr>
<th>Variables</th>
<th>Grade 1 (n=5)</th>
<th>Grade 2 (n=2)</th>
<th>Grade 3 (n=10)</th>
<th>P1</th>
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<th>P3</th>
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Grade 1, myxomas with both high tumor and high stroma MCP-1 expression; Grade 2, myxomas with only high tumor MCP-1 expression; Grade 3, myxomas with both low tumor and low stroma MCP-1 expression. P1, comparison between grade 1 and 2; P2, comparison between grade 1 and 3; P3, comparison between grade 2 and 3.

**Table 2** Correlation Between Extent of TP Expression and Clinicopathologic Features of Cardiac Myxoma

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Grade 1, myxomas with both high tumor and high stroma TP expression; Grade 2, myxomas with either high tumor or high stroma TP expression; Grade 3, myxomas with both low tumor and low stroma TP expression. P1, comparison between grade 1 and 2; P2, comparison between grade 1 and 3; P3, comparison between grade 2 and 3.
Correlations Between MCP-1 Expression, TP Expression, Microvessel Count, Macrophage Count and Clinicopathologic Features

As shown in Fig 3, the proportions of MCP-1-positive myxoma and stromal cells, and TP-positive myxoma and stromal cells significantly correlated with the increment of the microvessel count (p=0.023, p=0.016, p=0.012, and p=0.001, respectively). The proportions of MCP-1-positive myxoma and stromal cells significantly correlated with the proportion of TP-positive stromal cells (p=0.032 and p=0.017, respectively).

The mean microvessel count in myxomas with both high tumor and high stromal MCP-1 or TP expression was significantly higher than that in myxomas with low tumor and low stromal MCP-1 or TP expression (p=0.005 and p=0.005, respectively; Fig 4A,B).

Tumor size (≤55 mm in diameter) was significantly associated with high MCP-1 or TP expression (Table 1,2) but age and location had no discernible association with the expression of either factor. As shown in Fig 5, the microvessel count in small myxomas (≤55 mm in diameter) was significantly higher than that in large myxomas (p=0.015).

The CD68-positive macrophage count did not correlate with the microvessel count or tumor size. Although the statistical difference was not significant, myxomas with both high tumor and high stromal MCP-1 expression had a higher macrophage count than the other cases (Fig 6).

Discussion

Tumor growth is dependent on angiogenesis, which is regulated by various cytokines and growth factors produced by tumor cells and/or stromal cells. Because MCP-1 and TP are known to play important roles in the angiogenesis of different human tumors, we wanted to determine whether MCP-1 and TP are involved in the neovascularization of cardiac myxoma. To our knowledge, the current study is the first to detect the expression of both MCP-1 and TP, and to investigate their expression in relation to the angiogenesis and clinicopathologic features of cardiac myxoma.

Several lines of evidence have suggested that MCP-1 mediates the infiltration of macrophages into tumors and contributes to tumor angiogenesis. Transfection of tumor cells with the MCP-1 gene resulted in increased macrophage infiltration and neovascularization in a murine model.15 In human breast cancer, the concentration of MCP-1 has been shown to be significantly associated with macrophage accumulation and to correlate with the concentration of various angiogenic regulators such as VEGF, TP, and IL-8. Those findings indicate that MCP-1 can induce intratumor infiltration of macrophages, which may subsequently induce tumor angiogenesis through production of angiogenic factors. In the present study, we found that MCP-1, MCP-1 receptor CCR-2, and TP were overexpressed in cardiac myxoma. MCP-1 and TP were mainly located in myxoma cells and infiltrating macrophages, and the CCR-2 receptor was primarily expressed in infiltrating macrophages. Statistical analysis revealed that MCP-1 expression by tumor and stromal cells significantly correlated with an increased microvessel count and the mean microvessel count was significantly higher in myxomas with both high tumor and high stromal MCP-1 expression than in myxomas with low tumor and low stromal MCP-1 expression. Moreover, tumor and stromal MCP-1 expression also significantly correlated with stromal TP expression, and interestingly, in the tumor stroma, the locations that stained for CCR-2, TP- and CD68-positive cells in the serial sections were matched in most of the myxomas. Based on these findings, it is reasonable to speculate that in cardiac myxoma, MCP-1 plays an important role in tumor angiogenesis by attracting macrophages for the production of angiogenic factors such as TP. Furthermore, the fact that MCP-1 and CCR-2 were expressed in infiltrating macrophages also suggests an autocrine function of MCP-1 on macrophages via the CCR-2 receptor. Thus, in cardiac myxoma, MCP-1 expressed by infiltrating macrophages may function in an autocrine loop, taking part in macrophage infiltration and then mediating tumor angiogenesis.

Unlike a recent study of breast carcinoma,16 we failed to demonstrate a significant correlation between macrophage count and MCP-1 expression, and the macrophage count did not correlate with the microvessel count in cardiac myxoma. However, because we examined only 17 cardiac myxomas, it is possible the sample number was insufficient to reach statistical significance. In addition, because macro-
phages are a very complex and heterogeneous cell population and have several functions related to tumor biology, it is also possible that the production of MCP-1 alone may not be the only determinant of macrophage recruitment in cardiac myxoma. Other factors, such as chemokines produced by the host in response to the tumor, may also participate in macrophage recruitment and therefore further studies on a larger scale are required.

MCP-1 may also have a direct effect on tumor angiogenesis by inducing chemotaxis of endothelial cells, as evidenced by the detection of CCR-2 on the endothelial cell surface. In the present study, CCR-2 was also expressed on the endothelial cells of a few blood vessels. Although endothelial cells were not a major source of CCR-2 and in only 3 cases were a few CCR-2 positive endothelial cells noted, the endothelial migratory response to MCP-1 suggests that MCP-1-induced tumor angiogenesis might be partially dependent on the endothelial mechanism.

The importance of TP in tumor angiogenesis has been demonstrated in a variety of human tumors, but the localization of TP production varied according to the type of tumor. In patients with breast and gastric carcinoma, TP is produced mainly by tumor cells, which plays an important role in tumor angiogenesis. In contrast, Takahashi et al. reported that TP expressed by stromal infiltrating cells significantly correlated with microvessel density in colon carcinoma. From our present results, expression of TP was found not only in tumor cells, but also in stromal cells, and positively correlated with the microvessel count. Moreover, the microvessel count in myxomas with both high tumor and high stromal TP expression was higher than that in the other myxomas. Based on these observations, we conclude that in cardiac myxoma, TP expression in either tumor or stromal cells may contribute independently to tumor angiogenesis, and at the same time may contribute to promoting tumor angiogenesis.

In our previous study, we suggested that VEGF was inversely correlated with tumor size, and that the number of microvessels decreased as the myxoma grew. In the current study, high MCP-1 or TP expression was also observed in smaller myxomas and these had a significantly higher microvessel count than the larger tumors. These findings are unusual and indicate that angiogenic factors may play a more important role in neovascularization when the myxoma is smaller. Moreover, a differential significance of angiogenesis in the tumor progression is also suggested. In our study, small myxomas consistently had high neovascularization in the central part of the tumor, whereas in large myxomas the microvessels were frequently observed in the peripheral part of the tumor. It is reasonable to speculate that in cardiac myxoma, angiogenesis frequently occurs in the central part of the tumor in the early stage of tumor growth, but more frequently occurs in the tumor periphery as the myxoma enlarges. Although angiogenesis is essential for the growth of cardiac myxoma, the role of angiogenesis in these tumors may be different from that in malignant carcinomas. The unique growth conditions in the cardiac chamber may affect their angiogenesis.

In summary, MCP-1 and TP were detected in cardiac myxoma. MCP-1 may play an important role in tumor angiogenesis through production of angiogenic factors by recruited macrophages. TP expression in both tumor cells and stromal macrophages is important for the promotion of angiogenesis in cardiac myxoma. Together with our previous study, we conclude that cardiac myxomas produce VEGF, MCP-1 and TP, which induce the angiogenesis associated with tumor growth, especially in the early stage.

Acknowledgments

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