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INVESTIGATIVE REPORT

Serum Levels of Angiopoietin-2, but not Angiopoietin-1, are Elevated in Patients with Erythrodermic Cutaneous T-cell Lymphoma

Makiko KAWAGUCHI, Makoto SUGAYA, Hiraku SUGA, Tomomitsu MIYAGAKI, Hanako OHMATSU, Hideki FUJITA, Yoshihide ASANO, Yayoi TADA, Takafumi KADONO and Shinichi SATO
Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

Angiogenesis is a crucial process in the growth and progression of cancer, correlating with the metastatic potential of tumour cells. Angiopoietins are ligands for the endothelium-specific tyrosine kinase Tie2 receptor, which comprise 4 structurally related proteins, termed angiopoietin (Ang)-1, Ang-2, Ang-3 and Ang-4. The roles of Ang-1 and Ang-2 have recently been clarified as crucial in angiogenesis. In this report, we measured serum Ang-1 and Ang-2 levels in patients with cutaneous T-cell lymphoma (CTCL). Serum levels of Ang-2, but not Ang-1, in patients with Sézary syndrome were significantly higher than those in patch mycosis fungoides (MF), plaque/tumour MF, and healthy controls. In patients with CTCL, serum Ang-2 correlated with disease activity. Moreover, the numbers of Ang-2$^+$ cells in lesional skin of CTCL were significantly larger than those in normal skin. These results suggest that Ang-2 may have important roles in the development of CTCL. ***Key words: angiopoietin-2; angiopoietin-1; cutaneous T-cell lymphoma; CCL26; CCL27; mycosis fungoides; Sézary syndrome.***

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Makoto Sugaya, Department of Dermatology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: sugayam-der@h.u-tokyo.ac.jp

Mycosis fungoides (MF) and Sézary syndrome (SS) are the most common types of cutaneous T-cell lymphoma (CTCL) (1). MF is a T-cell malignancy that has a classically prolonged clinical course. Only limited cases progress over years through patch, plaque, and tumour stages, followed by lymph node and visceral involvement (2). SS is characterized by fever, erythroderma, lymphadenopathy, and leukaemic involvement, and usually has a rapid clinical course (3). Although the pathogenesis of CTCL is unknown, a variety of cytokines/chemokines are reported to be involved in development of the disease (4, 5).

Angiogenesis is a crucial process in the growth and progression of cancer, correlating with the metastatic potential of tumour cells (6, 7). Some clinical observations have indicated that tumour microvessel density, measured by CD34, CD31, or von Willebrand factor expression, is increased in some lymphoproliferative disorders. Higher tumour microvessel density and increased serum levels of proangiogenic factors, such as vascular endothelial growth factor (VEGF) or basic fibroblasts growth factor (bFGF), have been reported in chronic lymphocytic leukaemia, multiple myeloma, non-Hodgkin B-cell lymphomas, and CTCL (8, 9).

Angiopoietins are ligands for the endothelium-specific tyrosine kinase Tie2 receptor, which comprise 4 structurally related proteins, termed angiopoietin (Ang)-1, Ang-2, Ang-3, and Ang-4 (10, 11). The role of Ang-1 and Ang-2 has recently been clarified as a crucial molecule involved in angiogenesis alongside VEGF (10–12). Ang-1 and Ang-2 have been identified as ligands with opposing functions of the receptor tyrosine kinase. Ang-1 is constitutively expressed in pericytes and smooth muscle cells and acts in a paracrine agonistic manner by increasing tyrosine phosphorylation of Tie2, whereas Ang-2 acts primarily as an autocrine functional antagonist of Ang-1/Tie-2 (4, 5, 13–15). Under physiological conditions, Ang-1 has vasoprotective and anti-inflammatory actions, mediates vessel maturation, and maintains vessel integrity by the recruitment of peri-endothelial cells. Thus, low-level constitutive Tie2 activation by Ang-1 may be required in the adult to maintain the mature quiescent and non-proliferating phenotype of the vascular endothelium (12–14, 16, 17). By contrast, Ang-2 acts as a vessel-destabilizing cytokine, thereby playing an essential role in vascular remodelling. The function of Ang-2, however, is contextual. It facilitates angiogenesis in the presence of VEGF, but initiates vessel regression in the absence of proangiogenic activity (5, 13–15, 18).

The aim of this study was to evaluate serum levels of Ang-1 and Ang-2. Immunohistochemical staining for Ang-2 and CD31, a marker for endothelial cells, was also performed using lesional skin of patch MF, plaque MF, tumour MF, SS, and normal skin. Moreover, we evaluated correlation between serum levels of Ang-2 and those of C-C motif chemokine ligand (CCL) 11/eotaxin-1, CCL17/thymus and activation-regulated chemokine (TARC), CCL26/eotaxin-3, CCL27/cutaneous T-cell-attracting chemokine (CTACK), lactate dehydrogenase (LDH), immunoglobulin E (IgE), and soluble interleukin-2 receptor (sIL-2R).

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MATERIALS AND METHODS

Patients

Forty-five patients with CTCL (mean ± standard deviation (SD) age: 58.8 ± 16.2 years; 21 patch MF, 5 plaque MF, 9 tumour MF, and 10 SS) and 17 healthy control subjects (43.9 ± 18.6 years) were enrolled in this study. The diagnosis of MF and SS was based on clinical criteria as well as on histological and immunohistochemical assessment according to WHO classification (19). The 17 healthy controls had no history of allergy, CTCL, or any skin diseases. All samples were collected after informed consent during daily clinical practice. The medical ethics committee of the University of Tokyo approved all described studies, and the study was conducted according to the principles of the Declaration of Helsinki.

Enzyme-linked immunosorbent assay

Serum Ang-1, Ang-2, CCL11, CCL17, CCL26, CCL27 levels were quantified by Human Quantikine enzyme-linked immunoassay (ELISA) kits (R&D systems, Minneapolis, MN, USA). These assays employ the quantitative sandwich enzyme immunoassay technique. Optical densities were measured at 450 nm using a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The concentrations were calculated from the standard curve generated by a curve-fitting programme.

Immunohistochemistry

We performed immunohistochemical staining for Ang-2 and CD31 with lesional skin of patch MF ($n = 5$), plaque MF ($n = 5$), tumour MF ($n = 5$), SS ($n = 5$), and angiosarcoma ($n = 5$) as positive controls, and with normal skin ($n = 5$). Briefly, 5 μm-thick tissue sections from formaldehyde-fixed and paraffin-embedded samples were de-waxed and rehydrated. These sections were then stained with rabbit anti-human Ang-2 polyclonal antibody (Abcam plc, Cambridge, UK), and mouse anti-human CD31 monoclonal antibody (Dako, Glostrup, Denmark), followed by ABC staining (Vector Lab., Burlingame, CA, USA). Diaminobenzidine was used for visualizing the staining, and counterstaining with Mayer haematoxylin was performed, according to the manufacturers' instructions. The number of dermal individual Ang-2$^+$ cells and CD31$^+$ vessels per high-power field (HPF; × 200) were counted in skin lesions and healthy skin.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U* test and Student's *t*-test for comparison of 2 groups. For testing equality of population means among 3 or more groups, Kruskal-Wallis test and Scheffe's F test were used. Correlation coefficients were determined using the Spearman's rank correlation test. *p*-values < 0.05 were considered statistically significant.

RESULTS

Serum levels of Ang-1 and Ang-2 in patients with CTCL

Serum Ang-1 levels were not significantly different in patients with CTCL and in healthy controls (Fig.1a). There was also no significant difference in serum Ang-2 levels between the 2 groups. We subsequently examined serum Ang-1 and Ang-2 levels according to the types of skin lesions in CTCL (Fig. 1b). There were no significant differences in serum Ang-1 levels among patients with patch MF, plaque/tumour MF, and SS. Serum Ang-2 levels in SS patients were significantly higher than normal controls, patch MF, and plaque/tumour MF ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively).

Serum Ang-1 and Ang-2 levels before and after progression of CTCL

Serum Ang-1 and Ang-2 levels were measured in 3 CTCL cases (2 cases of tumour MF, 1 case of SS) before and after disease progression (Fig. 2). Disease activity was judged by skin conditions and serum markers, such as LDH and sIL-2R. In all 3 cases, serum Ang-2 levels increased after disease progression, while serum Ang-1 levels did not show any trend.

Correlation between serum levels of Ang-2 levels and clinical and laboratory data

We evaluated correlations between serum Ang-2 levels and eosinophil counts, serum levels of CCL11, CCL17, CCL26, CCL27, LDH, IgE, and sIL-2R, all of which were reported to be elevated in patients with CTCL (5, 20–26). Among the above disease markers, serum CCL26 and CCL27 levels correlated significantly with serum Ang-2 levels (Fig. 3).

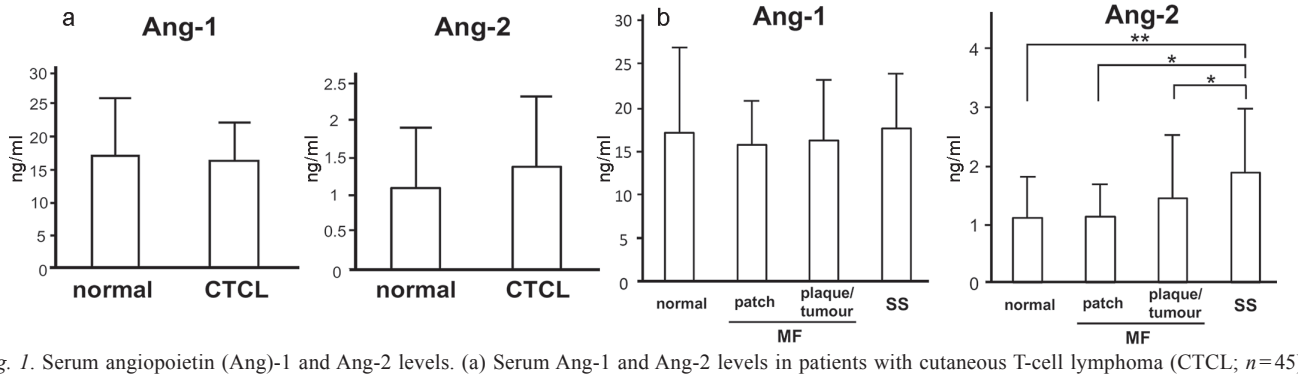

Fig. 1. Serum angiopoietin (Ang)-1 and Ang-2 levels. (a) Serum Ang-1 and Ang-2 levels in patients with cutaneous T-cell lymphoma (CTCL; $n = 45$) and healthy controls ($n = 17$). (b) Serum Ang-1 and Ang-2 levels in patients with various types of CTCL, including mycosis fungoides (MF) and Sézary syndrome (SS), and healthy controls. Each bar represents the mean ± SD of each group. *$p < 0.05$ and **$p < 0.01$.

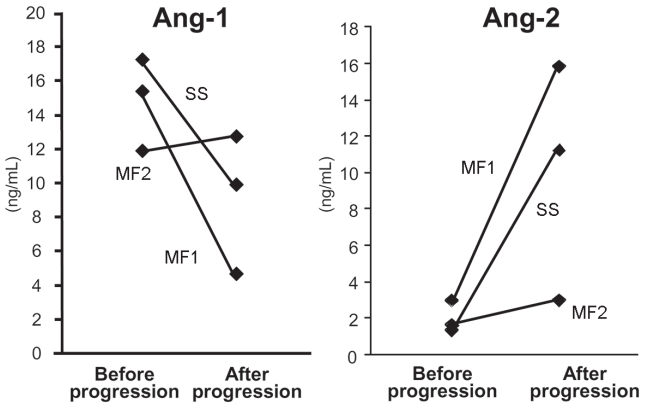

Fig. 2. Serum levels of angiopoietin (Ang)-1 and Ang-2 in cutaneous T-cell lymphoma before and after disease progression (time-span 5, 9 and 17 months for mycosis fungoides (MF)1, MF2 and Sezary syndrome (SS), respectively).

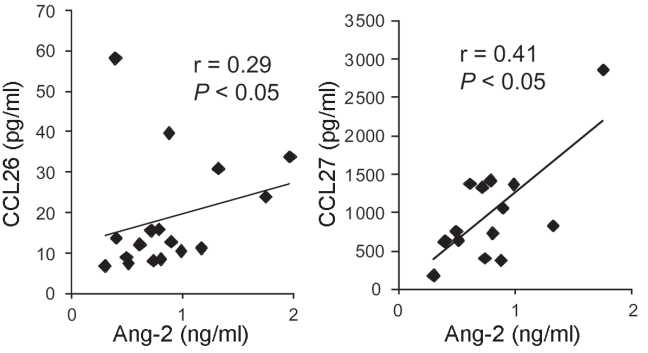

Fig. 3. Correlations between serum angiopoetin-2 levels and serum C-C motif chemokine ligand (CCL)26 ($n=17$) and CCL27 ($n=15$) levels in patient with cutaneous T-cell lymphoma.

Ang-2 expression and numbers of vessels in lesional skin of patch, plaque, tumour mycosis fungoides, Sezary syndrome, and normal skin

Immunohistochemical staining for Ang-2 was performed using normal skin, lesional skin of patch MF, plaque MF, tumour MF, SS, and angiosarcoma as a positive control (Fig. 4; left panel). In normal skin, Ang-2 signal was detected on and around the lumen of dermal vessels (Fig 4; *arrowheads*). In lesional skin of patch MF, some tumour cells around dermal vessels were also positive for Ang-2 (Fig. 4; *arrows*). In lesional skin of tumour MF and SS, tumour cells with large cytoplasm expressed Ang-2. We then stained the specimens for CD31, a marker for endothelial cells, in order to know whether Ang-2 expression was associated with increased blood vessels (Fig. 4; right panel). In normal skin, $CD31^+$ vessels were scarcely populated in the upper dermis. When counted in 5 cases in each group, the number of dermal $CD31^+$ vessels was increased in lesional skin of CTCL, especially in tumours (Fig. 5), which was consistent with a previous paper (27). The number of $Ang\text{-}2^+$ cells correlated significantly with that of $CD31^+$ vessels ($r=0.71$, $p<0.01$). Thus, endothelial cells and tumour cells in CTCL lesional skin expressed Ang-2, which was associated with enhanced angiogenesis, as previously reported in other malignancies (28–32).

DISCUSSION

Angiogenesis is involved in the development and progression of pathogenic processes in a variety of disorders, including diabetic retinopathy, psoriasis, rheumatoid arthritis, cardiovascular diseases, and cancer. With regards to the roles of angiogenesis in lymphoma, increased capillary proliferation in the lymph node biopsies of high-

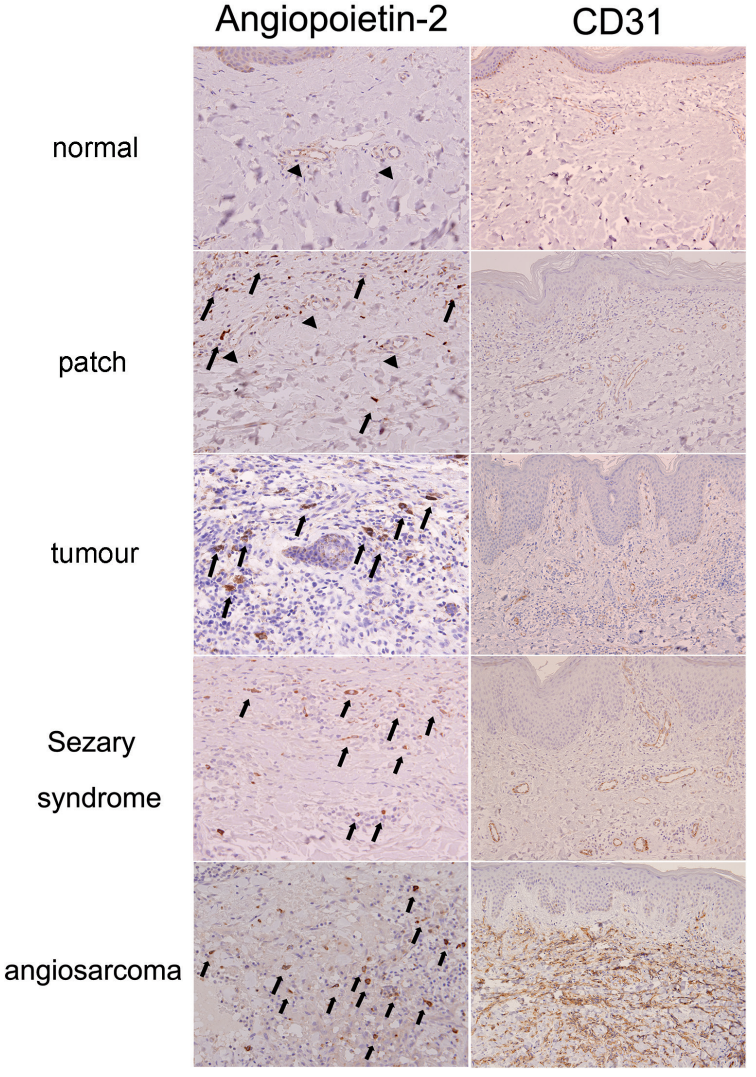

Fig. 4. Immunohistochemical staining for angiopoietin (Ang)-2 (original magnification × 400) and CD31 (original magnification × 100) using normal skin, lesional skin of patch mycosis fungoides (MF), plaque MF, tumour MF, and Sézary syndrome. Representative pictures from 5 cases in each group. Arrowheads indicate $Ang\text{-}2^+$ endothelial and arrows indicate $Ang\text{-}2^+$ tumour cells around vessels.

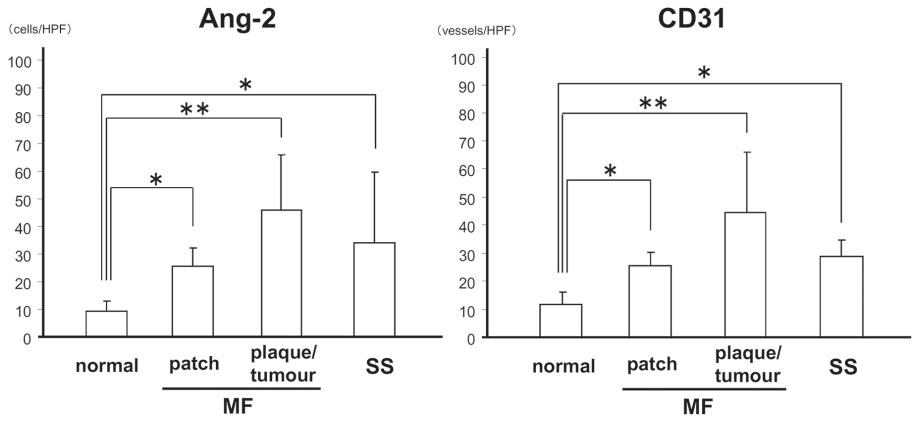

Fig. 5. Numbers of dermal angiopoietin-2$^+$ (Ang-2) cells and CD31$^+$ vessels per high-power field (HPF ×200) were counted in normal skin, lesional skin of mycosis fungoides (MF), and Sézary syndrome (SS). Each bar represents mean ± SD. *$p < 0.05$ and **$p < 0.01$.

grade non-Hodgkin's lymphoma (NHL) was reported (33). Tumour microvessel density has been shown to correlate with biological behaviour in nodal B-cell NHL (34, 35). On the other hand, tumour microvessel density was reported to be higher in the involved lymph nodes in patients with small lymphocytic lymphoma, but the number of blood vessels did not correlate with the grade of the tumour (36). Concerning angiogenesis in CTCL, increased angiogenesis and expression of matrix metalloproteinases 2 and 9 were reported to correlate with the progression of MF (27). Although precise mechanisms of angiogenesis in CTCL remain unclear, it is known that T cells, mast cells, and macrophages are capable of producing angiogenic factors (6). Therefore, increased capillary formation may be induced by lymphoma cells themselves and/or by tumour-associated host cells (9).

In this study, Ang-2, but not Ang-1, was elevated in patients with SS (Fig. 1). Similar findings were reported in cases with type 2 diabetes mellitus, where a selective increase in plasma levels of Ang-2 and soluble Tie-2, but not Ang-1, was accompanied by neovascularization and endothelial abnormalities (37, 38). Ang-2 levels were higher in acute congestive heart failure compared with chronic congestive heart failure, but there were no significant differences in Ang-1 levels between the groups (39). Similarly, serum Ang-2 levels were elevated in patients with multiple myeloma, while serum Ang-1 levels were not (40). Ang-2 mRNA was strongly expressed in lesional skin of angiosarcoma or Kaposi's sarcoma, while expression of Ang-1 mRNA was low (41). Taken together, Ang-2 plays more important roles than Ang-1 in some diseases with enhanced angiogenesis. Although Ang-2 was expressed in lesional skin of MF (Fig. 4, 5), serum levels of Ang-2 were elevated only in patients with SS. Thus Ang-2 may function only within lesional skin in MF. Circulating tumour cells in patients with SS may be the source of Ang-2 in sera. Serum Ang-2 levels increased after disease progression even in patients with MF (Fig. 2), which suggests that Ang-2 may be useful as a marker of disease activity for each patient, rather than a disease-specific marker.

In conclusion, because Ang-2 expression was enhanced in the sera and skin lesions of CTCL, angiogenesis may play a role in the growth of CTCL, raising the possibility of using angiogenesis inhibitors in CTCL therapy.

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The authors declare no conflicts of interest.

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