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## Original Paper

# Progression of Mycosis Fungoides is Associated with Changes in Angiogenesis and Expression of the Matrix Metalloproteinases 2 and 9

A. Vacca,[1] S. Moretti,[5] D. Ribatti,[2] A. Pellegrino,[1] N. Pimpinelli,[5] B. Bianchi,[5] E. Bonifazi,[3] R. Ria,[1] G. Serio[4] and F. Dammacco[1]

[1]Department of Biomedical Sciences and Human Oncology; [2]Institute of Human Anatomy, Histology and Embryology; [3]Institute of Dermatology; [4]Chair of Medical Statistics and Biometry, Institute of Hygiene, University of Bari Medical School, Piazza Giulio Cesare 11, I-70124 Bari; and [5]Institute of Dermatology, University of Florence Medical School, I-50100, Florence, Italy

**Changes in angiogenesis and expression of extracellular matrix-degrading enzymes have been substantiated during progression of solid tumours, whereas information on haematological tumours remains circumstantial. In this study, 57 biopsies of mycosis fungoides (MF), a haematological tumour of T-cell lineage, were investigated immunohistochemically for the extent of angiogenesis, and by *in situ* hybridisation for the expression of matrix metalloproteinases 2 (MMP-2, collagenase A) and 9 (MMP-9, collagenase B). The biopsies we grouped according to the stage of progression: patch → plaque → nodular (most advanced). The extent of angiogenesis, as microvessel area, of MF lesions as a whole was significantly higher than that of normal uninjured skin, used as a control. When the stages of MF progression were compared, the values of MF patch stage overlapped that of control skin, while values were significantly higher in the plaque stage and even higher in the nodular stage. In these stages, microvessels were widely scattered in the tumour tissue, in close association with tumour cells, and they frequently displayed arborisation and microaneurysmatic dilation. In contrast, in the patch stage microvessels were irregularly distributed around the tumour aggregates, and arborisation or dilated structures were only rarely seen. The expression of MMP-2 and MMP-9 mRNAs underwent significant upregulation in relation to advancing stage. Indeed, the upstaging was significantly associated with higher proportions of lesions positive for each mRNA or for both, and with lesions with the greatest intensity of expression for each mRNA. Besides tumour cells, the MMP-2 mRNA was expressed by microvascular endothelial cells of intratumour and peritumour vessels, and by fibroblasts which were especially abundant in the stroma adjacent to the tumour nodules. The MMP-9 mRNA was found to be present in a subset of tissue macrophages which were more frequently located in close vicinity to the tumour nodules. In contrast, in control skin, a weak positivity for the MMP-2 mRNA in very few microvascular endothelial cells and no signal for the MMP-9 mRNA were observed. These *in situ* data suggest that angiogenesis and degradation of the extracellular matrix occur simultaneously during MF progression. They imply that interaction between tumour cells and their microvasculature are all the more likely to occur during progression, occasionally with the contribution of tumour-associated stromal cells.** © **1997 Elsevier Science Ltd.**

**Key words: angiogenesis, matrix metalloproteinases, mycosis fungoides, tumour progression**
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Correspondence to A. Vacca.
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## INTRODUCTION

ANGIOGENESIS, THE formation of new microvessels, is fundamental for tumour progression in the form of growth, invasion and metastasis [1]. Microvessels promote growth because they convey nutrients and oxygen and remove catabolites, while endothelial cells secrete important paracrine growth factors for tumour cells [2, 3]. Microvessels facilitate invasion because endothelial cells at their tips secrete several extracellular matrix-degrading enzymes, which allow spread of tumour cells into and through the adjacent matrix [4]. They permit metastasis because an expanding endothelial surface increases opportunities for tumour cells to enter the circulation [5]. All these relationships have been found in solid tumours, such as lung, breast and colon carcinoma, and melanoma, where the microvessel density provides information on the biological aggressiveness and hence on the risk of metastasis or recurrence [6].

Additional events involved in progression comprise the secretion of matrix-degrading enzymes by tumour cells, including two matrix metalloproteinases (MMPs), namely type IV collagenases of 72 kDa (MMP-2 or gelatinase A) and 92 kDa (MMP-9 or gelatinase B). The MMP-2 and MMP-9 facilitate invasion and metastasis because they degrade type IV, V, VII and X collagens as well as fibronectin [4], which are important constituents of the interstitial stroma (collagens) and the subendothelial basement membrane (collagens and fibronectin) matrix [7]. Indeed, in carcinomas and melanomas, overexpression of both MMPs is associated with the invasive and metastatic phase, although not with *in situ* tumour or with the hyperplastic and normal counterpart [8, 9].

Knowledge of angiogenesis and MMPs in haematological tumours is fragmentary. Angiogenesis has been found to be correlated with tumour growth (S-phase fraction) in some lymphoproliferative diseases of B-cell lineage, such as multiple myeloma [10] and B-cell non-Hodgkin's lymphoma [11]; to be associated with leukaemic spreading and poor prognosis in multiple myeloma [12]; to be denser in the more aggressive lymphoproliferative diseases of T-cell lineage, such as T-cell immunoblastic and lymphoblastic lymphoma [13]. MMP-9 has been the only collagenase studied. It is frequently overexpressed by high-grade non-Hodgkin's lymphomas of B-cell lineage, where the intensity of expression correlates with systemic spreading and shorter survival [14], and it is secreted by several myeloid leukaemia cell lines following phorbol stimulation [15].

To investigate angiogenesis and expression of MMP-2 and MMP-9 in a well-defined model of progression of lymphoproliferative diseases, we investigated the extent of angiogenesis and overexpression of MMPs in distinct stages of clinical progression of mycosis fungoides (MF), a lymphoproliferative disease of T-cell lineage.

## MATERIALS AND METHODS

*Patients and skin specimens*

A total of 57 Caucasian patients who fulfilled the clinical, histopathological and immunohistological criteria for MF [16] were studied prior to therapy (Table 1). One 4-mm punch biopsy was taken from each patient, which was immediately fixed in 10% buffered formalin and embedded in paraffin. 6 μm adjacent sections were reserved for histopathological diagnosis, the evaluation of angiogenesis and MMP-2 and MMP-9 mRNA expression. On the basis of the clinical and histological data, the 57 biopsies were grouped according to the three steps of MF progression [16]. Step 1 included 16 patch stage MFs, i.e., tumours mainly located on the trunk and extremities and formed by nested lymphoid cells within a non-spongiotic epidermis and sparse lymphoid cells in the superficial dermis. Step 2 included 22 plaque stage MFs, i.e., tumours developing both on the trunk and extremities, as well as on the head and neck and consisting of nested lymphoid cells throughout the epidermis and the superficial dermis. Step 3 included 19 nodular stage MFs, i.e., exophytic tumours with or without ulceration anywhere in the body and formed by nested lymphoid cells involving the epidermis and the dermis extensively and in depth. Besides the clinical picture, steps 1–3 offer a clear representation of tumour progression because: (i) the clinical evolution from one step to the next is typical; and (ii) the tumour growth fraction (S-phase fraction) rises significantly in the transition from one step to the next.

*Table 1. Clinical and histopathological characteristics of the patients*

| | |
|---|---|
| Number of patients | 57 |
| Patch stage | 16 |
| Average age (median, range) | 57.9 years (57.5, 49–73) |
| Men/women | 6/10 |
| Cerebriform/blast cytology* | 16/0 |
| I/II/III/IV† | 6/10/0/0 |
| Plaque stage | 22 |
| Average age (median, range) | 67.1 years (68, 55–76) |
| Men/women | 10/12 |
| Cerebriform/blast cytology | 22/0 |
| I/II/III/IV | 8/14/0/0 |
| Nodular stage | 19 |
| Average age (median, range) | 61.5 years (61, 48–73) |
| Men/women | 8/11 |
| Cerebriform/blast cytology | 16/3 |
| I/II/III/IV | 3/10/4/2 |

*Blasts <30% [16]. †Staging system according to Bunn and Lamberg [17].

Twenty-four biopsies from the normal skin of sex- and age-matched persons undergoing surgical intervention for abdominal hernia or cholecystectomy were used as the controls.

The study was approved by the local ethics committee and all patients gave their informed consent.

*Assessment of angiogenesis*

Sections were deparaffinised by the xylene–ethanol sequence, washed in phosphate-buffered saline (PBS), and treated with 0.1% trypsin (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) in 0.1% calcium chloride. All blood vessels were highlighted by staining endothelial cells with a rabbit antiserum against the factor VIII-related antigen (Dako, Glostrup, Denmark) and the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique previously described [18]. Briefly, sections were sequentially incubated with the mentioned antiserum, an antiserum against rabbit IgG (from swine, Dako) and the APAAP complexes (Dako). Alkaline phosphatase activity was revealed by a freshly made solution of Fast Red (Sigma Chemical Co.) in Tris-HCl 0.05 M pH 7.6 (1 mg/ml) containing naphthol phosphate in *N,N'*-dimethylformamide (10 mg/ml) and levamisole 1 mM (Sigma Chemical Co.) After pink staining

developed, the sections were washed in the same buffer, counterstained in Gill's haematoxylin no. 2 (Polysciences Inc., Warrington, Pennsylvania, U.S.A.), dehydrated with ethanol–xylene and mounted in Eukitt.

Care was taken to select microvessels, namely capillaries and small venules, from all the stained vessels. A double-headed photomicroscope (Leitz Dialux 20, Leitz, Wetzlar, Germany) was used in the simultaneous identification by two investigators of these microvessels as endothelial cells, single or clustered in nests or tubes, clearly separated from one another and either without or with a lumen (not exceeding 10 μm). Each identification was agreed upon in turn. Microvessels were identified on 4–6 ×250 fields (×25 ocular and ×10 objective) covering almost the whole of each of two sections per biopsy, within a square mesh inserted in the eyepiece. The mesh was formed of 22 lines per side giving 484 intersection points, and defined an area of $12.5 \times 10^{-2}$ mm$^2$ per field (reference area), whereas each point covered an area of 64.5 μm$^2$. The area occupied by microvessels was estimated by using the direct planimetric method of 'point counting' [19] with slight modifications [20] applied to a computed image analysis system (Autocad 12, Autodesk Inc., Sausalito, California, U.S.A.). According to this method, the microvessel area equalled the sum of points that hit microvessels. Because of its very close points, the mesh allowed an accurate measurement of the microvessel area. Non-cellular areas due to necrotic and haemorrhagic foci were always omitted from the reference area, since they were devoid of microvessels and thus hampered comparison between sections. Points that hit non-cellular areas were subtracted from the 484 points of the reference area. Residual points defined cellular areas only and the microvessel area was expressed as its percentage. Thus, the measurement of the microvessel area fits the following equation: $x/(484 - p) = y/100$, where $x$ denotes the sum of points that hit microvessels, $p$ defines the sum of points that hit non-cellular areas and $y$ defines the microvessel area as a percentage. The mean ± 1 standard deviation (S.D.) were calculated per section, per biopsy and group of biopsies. CD31 and CD34 could not be used for endothelial cell staining, since they are shared with lymphocytes and (CD31) with macrophages and neutrophils [21].

In situ *hybridisation of MMP-2 and MMP-9 mRNA*

This was performed as described previously [22]. Deparaffinised sections washed in PBS were made permeable with 10 μg/ml of proteinase K (Sigma Chemical Co.) in $CaCl_2$ (2 mM) Tris (20 mM) for 5 min at 37°C, acetylated with 0.25% acetate in 0.1 M triethanolamine, washed in 2× saline-sodium citrate buffer (SSC), dehydrated in graded ethanol and air dried. They were hybridised overnight at 50°C with 5 μg/ml of two 5'-biotinylated oligonucleotides (Genenco Life Science, Florence, Italy), the first of 42 bases complementary to the ninth exon sequence 446–459 of the MMP-2 mRNA, the second of 48 bases complementary to the ninth exon sequence 445–460 of the MMP-9 mRNA [23]. As the hybridisation medium, 50% deionised formamide, 600 mM NaCl, 80 mM ethylenediamine tetraacetate (EDTA), 10 mM dithiothreitol, 1× Denhardt's solution and 100 μg/ml salmon sperm DNA was used. After washing in SSC (×2 to ×0.01) and PBS, sections were incubated overnight at 4°C with streptavidin–alkaline phosphatase conjugate (Promega Co., Madison, Wisconsin, U.S.A.). After revealing the alkaline phosphatase activity by Western blue stabilised substrate (Promega Co.), sections were mounted in buffered glycerine. They were then evaluated by two observers through the double-headed photomicroscope for the extent and intensity of the hybridisation signal within the tumour area. The signal was scored, relative to the background signal of RNAse-treated control sections hybridised with the same oligonucleotides, as follows: [1] weak (detectable), [2] moderate (easily visualised) or [3] strong. Samples were considered positive when >10% of tumour cells gave the signal. Negative samples, also scored as [0], were those with ≤10% of tumour cells displaying the signal. The cut-off was based on the finding that ≤10% of tumour cells seldom gave the signal in control sections.

*Statistics*

The significance of changes in microvessel area in the groups of normal skin, patch, plaque and nodular stage MF was assessed with Fisher's Exact test and the non-parametric Kruskal–Wallis test. Because this showed that some changes were significant, it was followed by Duncan ($t$), Bonferroni ($t$) and Wilcoxon tests to compare groups two by two. A linear regression test was applied to relate the percentages of the MMP-2- and MMP-9-positive and -negative MF tissues with steps in tumour progression. The chi-squared test was split into the linear and the residual component according to Cochran. Data were computed with the Statistical Analysis Software (SAS, SAS Institute Inc., Cary, North Carolina, U.S.A.).

## RESULTS

*Angiogenesis*

The values of microvessel area in tissues from normal uninjured skin (control) and from MF lesions as a whole and grouped by progression stages are shown in Table 2. The comparison between groups revealed statistically significant differences (chi-squared = 47.49, df = 3, $P < 0.01$; $F = 42.81$, $P < 0.01$). When differences were sought between groups, significantly higher values were found in overall MF lesions compared with control skin ($P < 0.01$). When each MF stage was compared to control skin, no significant difference was observed in the patch stage, whereas

*Table 2. Microvessel area in control skin and mycosis fungoides clinical progression stages*

| | Control skin | Stage | | | |
|---|---|---|---|---|---|
| | | All | Patch | Plaque | Nodule |
| | $n = 24$ | $n = 57$ | $n = 16$ | $n = 22$ | $n = 19$ |
| Mean ± SD | 0.69 ± 0.35 | 2.25 ± 1.5* | 0.85 ± 0.4 | 2.1 ± 0.83† | 3.59 ± 1.56‡ |
| (Median, range) | (0.65, 0–1.5) | (1.85, 0.4–7.4) | (0.75, 0.4–1.85) | (1.95, 1–4.5) | (3.3, 1.2–7.4) |

*$P < 0.01$ compared to control skin; †$P < 0.01$, compared to patch stage; ‡$P < 0.01$ compared to plaque stage.

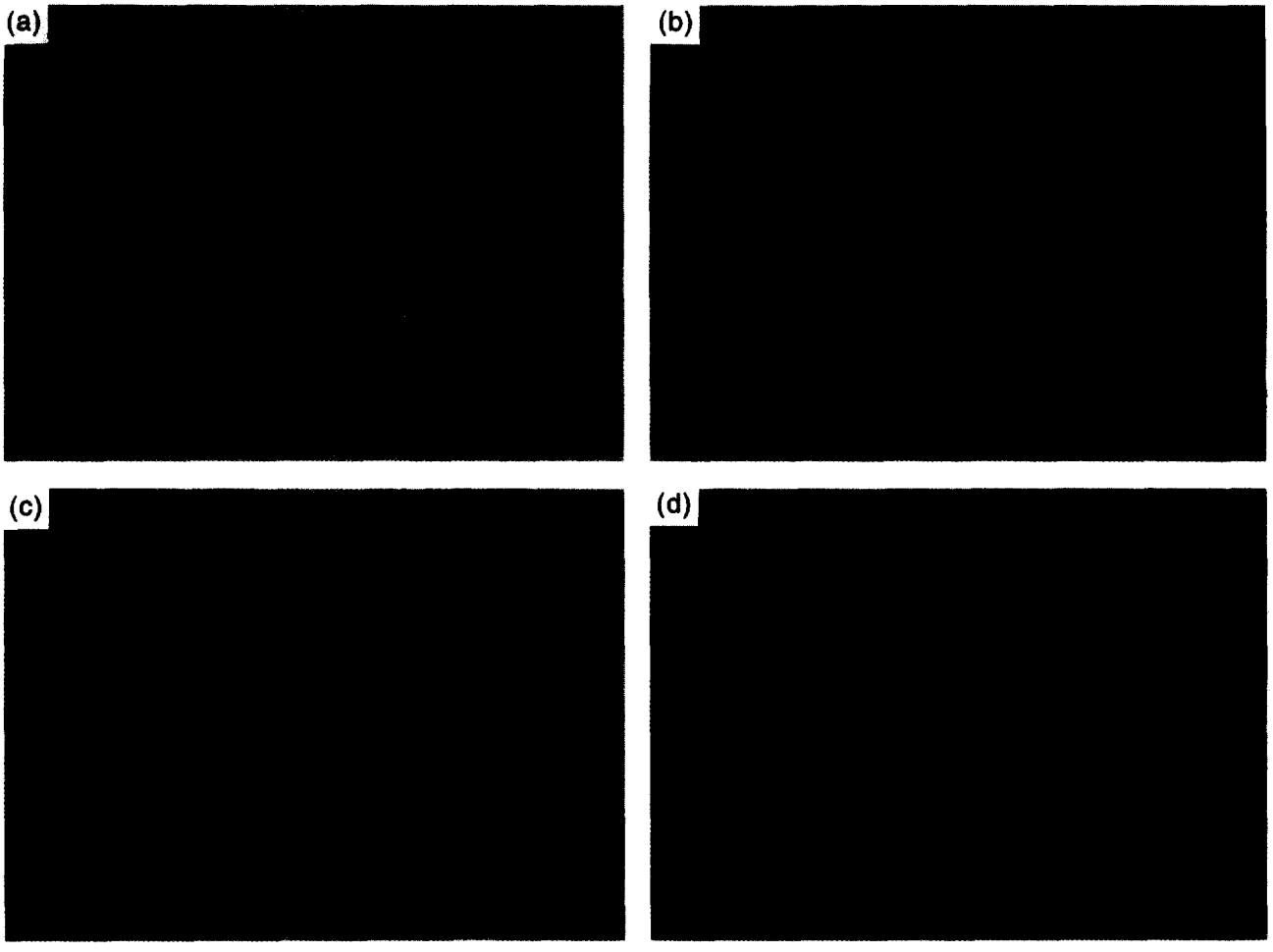


**Figure 1. Staining of mycosis fungoides: (a) patch, (b) plaque and (c) nodular stages in the skin, and of (d) normal skin with factor VIII-related antigen (×160 fields). The microvessel areas were 0.7%, 2.2%, 4.3% and 0.9%, respectively.**

both plaque and nodular stages had significantly higher values ($P < 0.01$). The interstage comparisons showed that the microvessel area increased in parallel with progression: the value in the plaque stage was significantly higher ($P < 0.01$) than the value for the patch stage and the value for the nodular stage was significantly higher ($P < 0.01$) than that of the plaque stage (Figure 1). In the MF lesions, the area was correlated neither with cytology nor with the Bunn and Lamberg staging, although a higher number of patients is needed for a more definite conclusion.

Histologically, in all tissues microvessels were mainly seen as endothelial cells clustered in nests or tubes, transversally or longitudinally sectioned, with or without a small lumen (2–4 μm in diameter). In the control skin, these vessels were very rare and especially confined to the superficial dermis. In the patch stage, they were mostly located around the tumour aggregates (Figure 1(a)). By contrast, in the plaque and nodular stages they were widely distributed throughout the tumour tissue and were closely interwoven with tumour cells (Figure 1(b) and (c)); they often occurred in large numbers in the dermis adjacent to tumour aggregates. Also, they frequently showed arborisation as well as dilated, microaneurysmatic segments, both of which were rare in the patch stage and absent in the control skin.

*Matrix metalloproteinases*

The percentages of MF lesions expressing MMP-2 or MMP-9 mRNAs, and coexpressing the mRNAs, are shown in Table 3. All percentages increased in parallel with tumour progression. Indeed, compared to the patch stage, the plaque stage and even more the nodular stage gave sig-

*Table 3. Expression and coexpression of mRNA for matrix metalloproteinases 2 (MMP-2) and 9 (MMP-9) by stages of clinical progression*

| | Stage | | |
|---|---|---|---|
| mRNA of | Patch $n = 16$ | Plaque $n = 22$ | Nodule $n = 19$ |
| MMP-2 | | | |
| Present | 37.5* | 59.1 | 78.9 |
| Absent | 62.5 | 40.9 | 21.1 |
| MMP-9 | | | |
| Present | 12.5 | 31.8 | 52.6 |
| Absent | 87.5 | 68.2 | 47.3 |
| MMP-2 and MMP-9 | | | |
| Present | 6.3 | 22.7 | 42.1 |
| Absent | 93.7 | 77.3 | 57.9 |

*Data expressed as percentages of lesions.

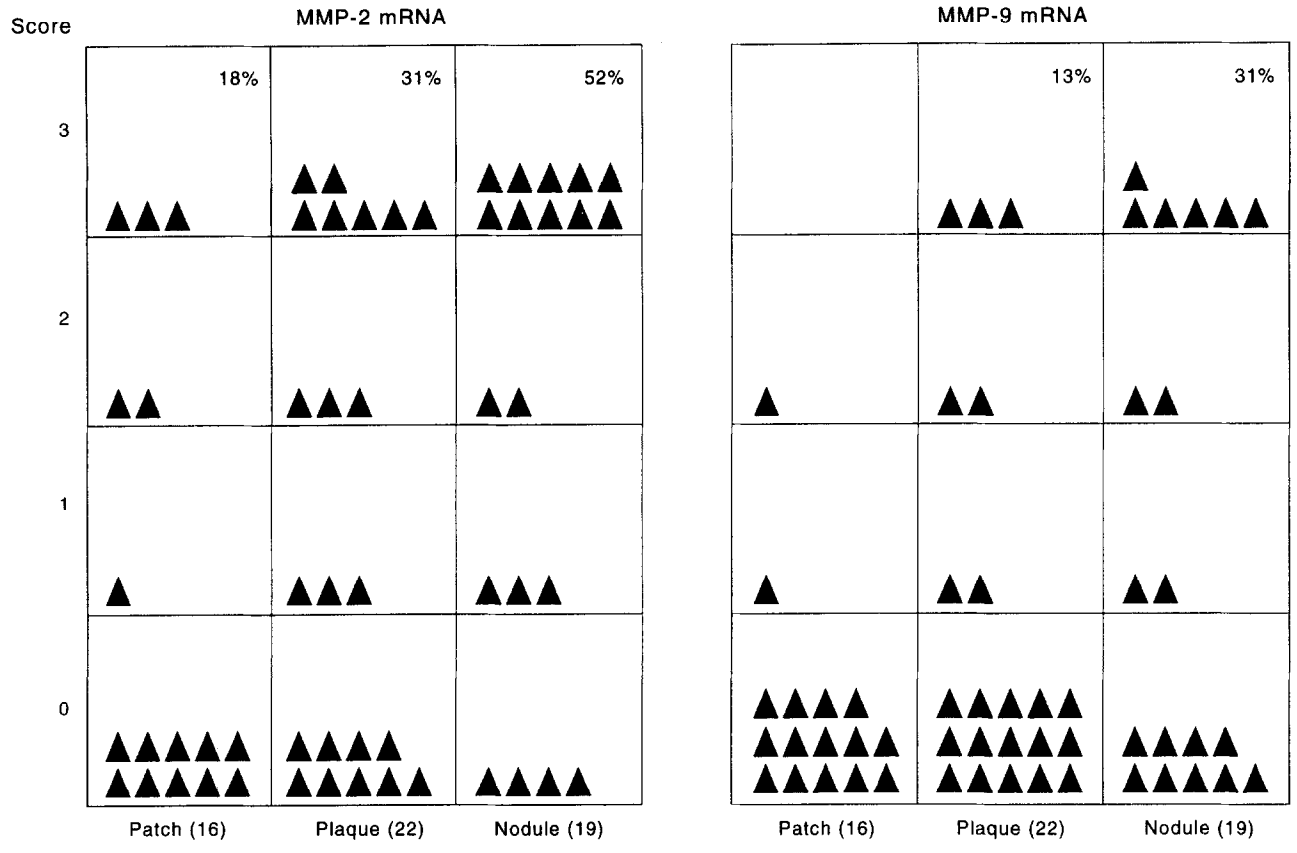


**Figure 2. Intensity score for *in situ* hybridisation of matrix metalloproteinase 2 (MMP-2) and MMP-9 mRNA in mycosis fungoides at different stages of progression. Each triangle represents a single lesion. The percentages of lesions of the highest score are reported. Number of lesions between brackets.**

nificantly higher percentages of lesions expressing the MMP-2 mRNA (chi-squared linear = 6.1, $P < 0.05$), the MMP-9 mRNA (chi-squared linear = 6.3, $P < 0.05$) and both mRNAs (chi-squared linear = 6, $P < 0.05$).

The intensity of expression of MMP-2 and MMP-9 mRNA was assessed by the following score system: 1 (weak intensity), 2 (moderate) or 3 (strong) (Figure 2). The frequency of lesions scoring 1 or 2 remained fairly constant in all stages. In contrast, the highest score for both mRNAs occurred with a significantly higher frequency in proportion to the upstaging. For MMP-2, it was detected in 18% of lesions of the patch stage, and in 31% and 52% of those of the plaque and nodular stage, respectively (chi-squared total = 6.42; chi-squared linear = 6.3, $P = 0.05$). For MMP-9, it was absent in the patch stage, whereas it was found in 13% and 31% of lesions of the plaque and nodular stage, respectively (chi-squared total = 7.61; chi-squared linear = 7.5, $P = 0.05$).

Histologically, the MMP-2 and MMP-9 mRNAs produced a cytoplasmic staining pattern (Figure 3). In all MF stages, both mRNAs were not expressed by the entire tumour cell population, but by single cells or nests randomly scattered in the tumour area, which resulted in a very heterogeneous, 'ground-glass'-like staining pattern (Figure 3). These mRNAs were generally not expressed by identical subpopulations of tumour cells. The location of positive cells was independent of the skin structures, such as hair follicles, vessels or glands, or of the skin layer.

In all stages, the mRNAs were also found in some stromal cell populations. MMP-2 mRNA was expressed by endothelial cells of intratumour and peritumour microvessels (Figure 3) and by fibroblasts in all layers of the skin, although these cells were especially located in the stroma within or around the tumour aggregates (Figure 3). MMP-9 was expressed by relatively few large cells frequently clustered in the stroma nearby the tumour aggregates. These cells were identified as a subpopulation of tissue macrophages by immunostaining of adjacent sections with a monoclonal antimacrophage antibody (CD68; data not shown). In contrast to the MF lesions, the control skin displayed expression of the MMP-2 mRNA in a few microvascular endothelial cells, whereas no MMP-9 mRNA could be demonstrated in any of the specimens.

## DISCUSSION

Our findings indicate that angiogenesis, measured as microvessel area, and the expression of matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9) mRNAs in tissues from MF change significantly, depending on the stage of tumour progression. The area was small (overlapping that of normal skin) in the patch stage, it expanded significantly in the plaque stage and even more in the nodular stage. The results provide the first demonstration that angiogenesis increases in proportion to upstaging of MF.

Although we have carried out experiments *in situ* and not functional observations, it seems reasonable to infer that the new vessels are induced by tumour cells, and that their

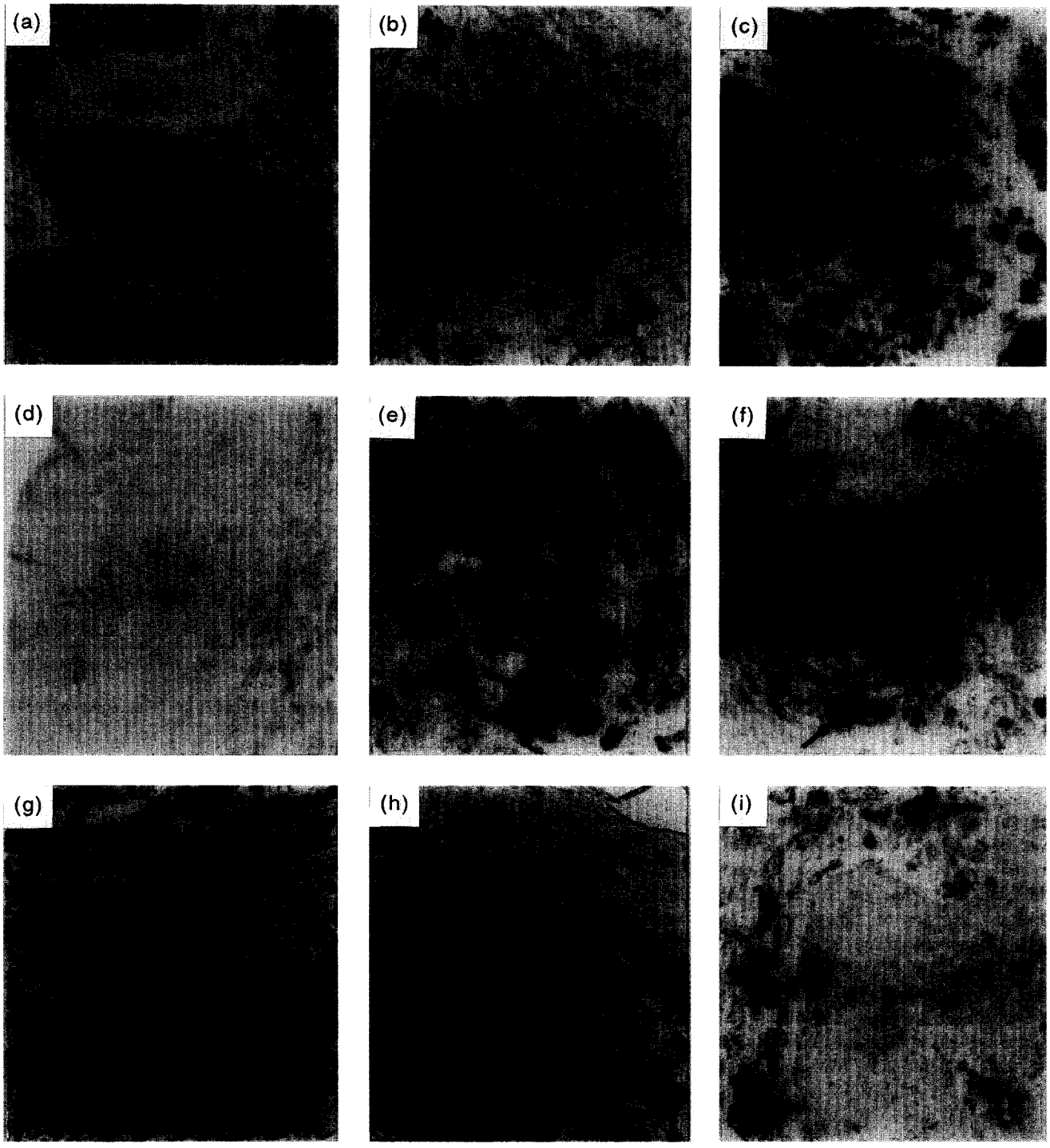


**Figure 3. (a)–(d) Nodular stage mycosis fungoides: (a) expression of matrix metalloproteinase-2 (MMP-2) mRNA and (b) and (c) of MMP-2 and MMP-9 mRNA, respectively, in adjacent sections. A colorimetric signal is present in most tumour cells, producing a 'ground-glass'-like picture; note in (a) the simultaneous expression of intratumour microvessels by endothelial cells (arrows); (d) the negative control for MMP-9. (e)–(g) Plaque stage mycosis fungoides: expression of MMP-2 mRNA by (e) scattered and (f) clustered tumour cells in superficial dermis; note in (f) the strongly positive peritumoral microvessels (arrows); (g) expression of MMP-9 mRNA by clustered tumour cells in the lower dermis. (h) Patch stage mycosis fungoides: intense signal for MMP-2 mRNA is present in scattered tumour cells in the superficial dermis. (i) Nodular stage mycosis fungoides: intense signal for MMP-2 mRNA is observed in spindle-shaped fibroblasts (arrows) within the tumour cells. Original magnifications: (a), (d) ×200; (b), (c), (f), (g) ×400; (h), (i) ×350; (e) ×1000.**

angiogenic ability is enhanced in the more advanced tumour progression stages. Angiogenesis could be stimulated either directly or indirectly, after the tumour cells have recruited inflammatory cells (macrophages, lymphocytes, mast cells) stimulating them to secrete their own angiogenic factors [24]. This hypothesis is supported by the finding that MF cells express the functional Th2 phenotype, more frequently in the plaque and nodular stages, and thus secrete IL-4 [25], which acts as a potent growth factor on microvascular endothelial cells [26]. However, MF cells in some instances [27] and infiltrating T lymphocytes [28] can secrete IL-2, which in turn stimulates other inflammatory cells to secrete

angiogenic factors [29]. Human dermal microvascular endothelial cells have also been shown to proliferate [30] and migrate [31], which are necessary functions in the development of angiogenesis [32]. Finally, skin angiogenesis has been demonstrated in a non-neoplastic disease, such as psoriasis [33].

The fact that angiogenesis proceeds in step with MF clinical progression suggests that it does favour progression. Indeed, neovessels promote growth [2, 3], which seems to explain why the S-phase fraction and morphologically the number of blasts rise in the transition from the patch to the nodular stage [16]. Neovessels favour invasion and metastasis [1, 4, 5], which could explain why loss of epidermotropism and invasion of lymph nodes and parenchymal organs are frequently observed in plaque-, and even more, in nodular-stage patients [16].

To our knowledge, no previous reports are available on the histological localisation of MMP-2 and MMP-9 or their mRNA in MF. Here we show that MMP-2 and MMP-9 mRNAs are significantly upregulated from patch to nodular stage: the increase in the frequency of lesions positive for MMP-2 and MMP-9 with advancing stages was accompanied by an increasing number of lesions with the strongest expression intensity. The data suggest that these MMPs are produced more frequently and in greater quantities with advancing MF progression and that, accordingly, a more intense degradation of interstitial stroma and subendothelial basement membrane take place with progression.

Hence, the data could provide an explanation for the greater dissemination and deepening of MF cells into the dermis as the disease progresses from patch to nodular stage, and account, together with denser angiogenesis, for the spreading of MF cells in the lymph nodes via the new lymphatic vessels, and parenchymal organs via the new blood vessels, in the plaque- and, more often, in the nodular stage. Finally, in all the MF lesions investigated, certain stromal cells expressed the MMPs mRNA. The signal for MMP-2 mRNA was detected in microvascular endothelial cells and fibroblasts, whereas that of MMP-9 mRNA was seen in a subset of macrophages. By contrast, in the control skin, the MMP-2 mRNA was expressed by few microvascular endothelial cells only, and no signal for MMP-9 mRNA could be demonstrated.

These results provide evidence that mRNAs for the two MMPs in stromal cells characterise the malignant condition, and agree with those reported in solid tumours [34, 35]. It is conceivable that stromal cells, probably recruited and activated by MF cells, produce the MMPs, thus participating in the degradation of the extracellular matrix, and contributing to the tumour dissemination. Together with the findings in solid tumours, our data suggest that regulation of extracellular matrix degradation during tumour progression is the result of a concerted action, not only of several proteolytic enzyme systems, but also of several cell types, including both malignant and non-malignant cells in tumour stroma.

In conclusion, our *in situ* data show that angiogenesis and overexpression of MMP-2 and MMP-9 mRNAs occur simultaneously during MF clinical progression. This suggests more opportunities for MF cells to disseminate locally and to enter the circulation and spread systemically in parallel with tumour progression. Therapy with anti-angiogenic agents and/or tissue inhibitors of metalloproteinases can be envisaged as possible future development [36].


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