

Original article

Mast cells and vasculature in atopic dermatitis – potential stimulus of neoangiogenesis

Background: Atopic dermatitis skin lesions are characterized by inflammatory changes and epithelial hyperplasia requiring angiogenesis. As mast cells may participate in this process via bidirectional secretion of tissue-damaging enzymes and pro-angiogenic factors, the present study aimed to assess the occurrence and possible function of mast cells in the papillary dermis and in epidermal layers of atopic dermatitis lesions.

Methods: Semi-thin and serial sections in combination with immunohistochemistry, histochemistry and proliferating cell nuclear antigen (PCNA)-activity assays were used and related to epidermal thickness and targeted gene expression studies.

Results: Mast cells were located in the papillary dermis and migrated through the basal lamina into the epidermis of atopic dermatitis lesions. An increased PCNA-activity in cells of superficial epidermal layers indicated an activation of keratinocytes and stimulation of endothelial growth. Only ~30% of the papillary mast cells stained with the tryptase were toluidin-blue-positive, and ~80% were chymase positive. A high number of mast cells expressed c-kit. Most papillary and epidermal mast cells were localized close to endothelial cells. Vascular expression of endoglin (CD105) demonstrated neoangiogenic processes. Mast cells stimulation led to the expression of proangiogenic factors. Also, gene expression of tissue-damaging factors such as matrix metalloproteinases was increased.

Conclusions: These data suggest that in atopic dermatitis, mast cells are abundantly localized close to and within the epidermis where they may stimulate neoangiogenesis. Via the new vessels, inflammatory cells, together with complement components and antibodies, can be transported to the epidermis to aid in the defense against environmental antigens and to maintain chronic inflammation.

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Angiogenesis encompasses the formation of vascular tissues from pre-existing vessels, i.e. capillaries and post-capillary venules (1). It is the result of endothelial sprouting or nonsprouting (intussusceptive) microvascular growth (2). In the human skin, the vascularization consists of a deep vascular plexus which is situated at the dermal-subcutaneous border and a superficial vascular plexus which is found directly beneath the epidermal layer. Both vascular systems are densely interconnected and account for the control of immune cell influx, supply of nutrients, and thermoregulation.

Under both physiological and pathological conditions, angiogenic stimuli are believed to originate rather from the epidermis than the dermis (3) as the physiologically avascular epidermis requires mechanisms to meet the high metabolic demands associated with inflammatory or hyperproliferatory processes. Proangiogenic factors may also be produced by mast cells (4–6) but a direct link between the epidermis as origin of

angiogenic stimuli and mast cells as an angiogenesis-orchestrating cell type has not been established so far. Also, there has only been a preliminary study published on the possible occurrence of mast cells in the epidermis (7). Therefore, we addressed the presence and possible function of papillary and epidermally located mast cells in atopic dermatitis. This chronic inflammatory skin disease represents the initial clinical manifestation of allergic disease that often precedes allergic asthma or rhinitis and food allergies and has a proangiogenic background.

Methods

Tissues

Skin biopsies taken for diagnostic purposes from 27 patients with atopic dermatitis were included in the present study after informed consent and official approval. The atopic dermatitis diagnosis was

based on the criteria of Hanifin and Rajka, as confirmed on histopathology, with characteristic features of inflamed chronic eczematous lesions in all cases (8). Control tissues were obtained from normal skin of 20 patients who were biopsied for localized benign skin disease. Lack of pathology in these control specimens was confirmed by routine histology. All staining procedures were performed on paraffin-embedded tissues.

Antibodies

The following monoclonal or polyclonal antibodies were used: mast cell tryptase [AA1(9), provided by Dr A. Walls, Southampton, UK], mast cell chymase [CC1 (10)], c-kit (11), CD31, CD105, and proliferating cell nuclear antigen (PCNA) (purchased from DAKO, Hamburg, Germany), collagen type IV (DAKO), and matrix metalloproteinase (MMP)-2 (Biotrend, Cologne, Germany).

Toluidin-blue-staining

Carnoy-fixed paraffin-embedded tissue sections were stained with toluidin-blue solution (0.2% in 0.5 N HCl) overnight and afterwards washed and dehydrated with ethanol and xylol prior to mounting, as described previously (12, 13).

Histochemical staining methods

Immunocytochemistry using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique was performed on 5 µm paraffin sections, as described before (4, 12). Antibody reactivity was evaluated in a blinded fashion, counting at least 100 cells, recording distinctly stained cells only.

Fluorescence immunohistochemistry

Parallel expression of two proteins was detected either in serial sections or by double staining, as previously described (14). When double labeling was performed, suitable secondary antibodies coupled to different fluorochromes were applied after blocking with 5% skim milk-phosphate buffer saline (PBS), pH 7.4, and incubation with primary antibodies overnight: Cy3-conjugated goat anti-rabbit IgG serum (1 : 250 dilution in skim milk-PBS) and Cy2-conjugated donkey anti-mouse IgG (1 : 100 dilution) for 1 h at room temperature (Dianova, Hamburg, Germany). Cell nuclei were stained with 4',6-diamino-2-phenylindole (DAPI; Sigma, Deisenhofen, Germany) diluted 1 : 100 in PBS.

Semithin sections

For ultrastructural preembedding histochemistry, 20-µm-thick slices were generated in a vibratome and deparaffinized. After epon embedding, 1 µm sections were cut and stained with 1% methylene-blue solution.

Targeted gene expression profiling

After localization of mast cells in the epidermis, targeted gene expression profiling was used to identify potential pro-angiogenic factors released by mast cells upon pathophysiological stimulation. For these studies, the established human mast cell line 1 (HMC-1) was used (kindly provided by Dr J.H. Butterfield,

Rochester, MN, USA). The cells were previously established from leukemic patients (15) and express mast cell characteristic markers (12). HMC-1 cells were cultured routinely as described previously (12, 16). For mild stimulation to simulate the pathophysiological situation, HMC-1 cells were treated for 4 h with phorbol myristate acetate (PMA, Sigma) at a low concentration of 1 ng/ml. RNA isolation for the expression analysis and reverse transcriptase-polymerase chain reaction was performed as described previously (17). The targeted profiling analysis was performed by Incyte Genomics and BioCat GmbH (Heidelberg, Germany) as previously described (18, 19). The expression levels were measured as signal/background ratio as described previously (19, 20).

Statistics

Statistical significance was analyzed using the unpaired two-tailed *t*-test.

Results

Epidermal and papillary localization of mast cells

Immunohistochemical staining using an antibody against tryptase revealed tryptase-positive mast cells in the upper and deeper dermis of chronic lesions from patients with atopic dermatitis and in normal skin. Out of the specimen obtained from 27 patients, biopsies from five patients were found to have mast cells located in the epidermis (Fig. 1). In these lesions, epidermal mast cells were not

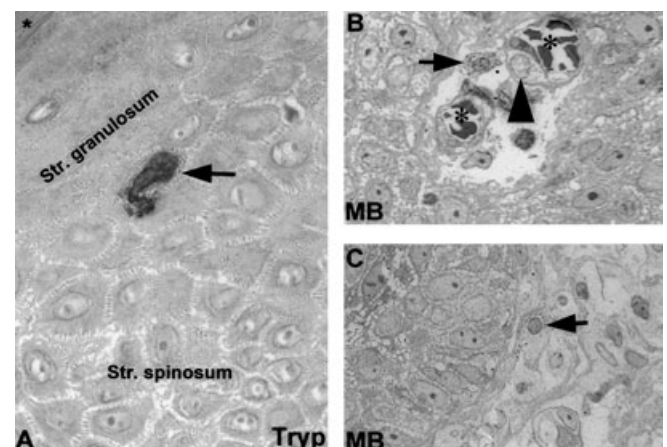


Figure 1. Epidermal and papillary localization of mast cells. Immunohistochemical staining using an antibody against tryptase was performed in lesional biopsies from atopic dermatitis patients and tryptase-positive mast cells are localized up to the stratum granulosum (arrow in A). Asterisk indicates beginning of stratum corneum. Semi-thin section techniques were applied in combination with methylene blue (MB) staining and demonstrated mast cells (arrow in B) in the epidermis close to vascular structures with endothelial cells (arrowhead). Mast cells were also present near the basal lamina (arrow in C). Original magnification: $\times 400$.

only in close contact to the basal lamina, but also in more superficial layers such as the stratum spinosum and the stratum granulosum (Fig. 1). In semi-thin sections stained with toluidin-blue, papillary and epidermal mast cells were often located in contact with endothelial cells (Fig. 1). Morphometry was used to analyze the different stages of mast cell penetration into the epidermis and four different grades were found, ranging from mast cells located in the papillary dermis near the basal lamina (grade I), mast cells at the basal lamina (grade II), mast cells at the epidermal side of the basal lamina (grade III) and finally mast cells located within the epidermis (grade IV), with signs of tissue degradation.

The basal lamina was stained using collagen type IV antibodies to identify intra-epidermally, nonpapillary localized mast cells. It was found that next to papillary areas, identified by circumferential staining for collagen type IV, also mast cells were present in epidermal areas not encircled by collagen type IV-positive basal lamina (Fig. 2).

Phenotype of papillary and epidermal mast cells

To identify the phenotype of papillary and epidermal mast cells which were found in lesional biopsies, immunohistochemistry using specific antibodies was applied and led to abundant signaling. All three markers against tryptase, chymase and c-kit were present in epidermal, papillary and other dermal mast cells. The comparison of tryptase and chymase contents of nonepidermal mast cells in normal and lesional atopic dermatitis skin showed characteristic differences. Serial sections of the lesional biopsies were submitted to immunohistochemistry to determine the presence of the markers in papillary and epidermal mast cells. It was found that all combinations of markers (c-kit-tryptase; tryptase-toluidin-blue, and tryptase-chymase) were present in the population. There were more c-kit- positive than tryptase-positive mast cells in the higher epidermal layers. Also, only about 1/3 of the tryptase-positive cells were toluidin-blue-positive indicating a high cellular secretory activity.

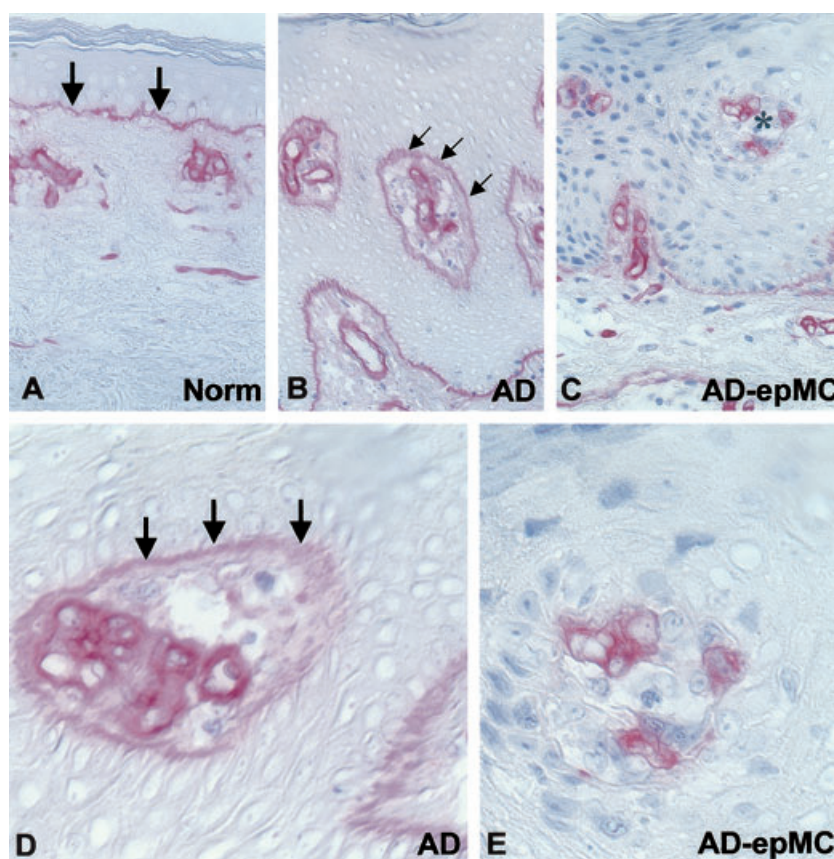


Figure 2. Epidermal and papillary area differentiation. Basal lamina staining using a collagen type IV antibody was performed to rule out that mast cells were only localized to cross-sectioned papillary areas in the epidermis. In normal skin (Norm), papillary areas were usually not present in the epidermis (A). In atopic dermatitis (AD), papillary areas were found in the epidermis as demonstrated by surrounding basal lamina [arrows in (B), high magnification in (D)]. In biopsies with epidermal mast cells (AD-epMC), areas were present with mast cells without surrounding basal lamina [asterisk in (C), high magnification in (E)]. Original magnification $\times 100$ (A)–(C), $\times 400$ (D) and (E).

Approximately 80% of the tryptase-positive cells were chymase-positive.

Relation to vascular structures and activity

To characterize the relationship between papillary and epidermal mast cells to vascular structures, immunohistochemical staining of normal skin and lesional skin from patients with atopic dermatitis was performed, using endothelial marker antibodies. It was found that numerous mast cells were in close contact with endothelial cells. In contrast to normal skin, CD31-positive endothelial cells were found to be sprouting from the papillae up towards the stratum granulosum only in lesional skin of the five patients with epidermal mast cell localization (Fig. 3). In the epidermis of these patients, there were also endothelial cells positive for CD105, a marker of vascular proliferation (Fig. 3). Fluorescence-double staining for the mast cell marker c-kit and CD31 in the lesional atopic dermatitis papillary area and epidermis revealed a juxtaposition of mast cells and endothelial cells (Fig. 3).

To identify the proliferative activity of the lesional epidermis in comparison to normal skin, sections were submitted to immunohistochemistry for PCNA, and an abundant signaling was found in the epidermis of atopic dermatitis lesions (Fig. 3). In this respect, not only endothelial cells, but also keratinocytes in superficial layers displayed proliferation activity. By contrast, only a few epidermal cells of the basal layers were PCNA-positive in normal skin (Fig. 3).

Epidermal wall thickness

To clarify whether the stimulus of angiogenesis and mast cell influx into the epidermis in biopsies with epidermal mast cell localization was the increased thickness of the epidermal layer, the epidermal thickness was assessed in the different groups. There was a significant difference in epidermal thickness between normal skin and lesional skin, with that in atopic dermatitis being threefold higher (Fig. 3). However, subgroup analysis of the atopic dermatitis group did not reveal a significant difference in the epidermal thickness of atopic dermatitis patients with and without epidermal mast cell localization (Fig. 3).

HMC-1 targeted gene expression profiling

After the demonstration of mast cells in the lesional epidermis of patients with atopic dermatitis, differential gene expression studies were carried out as an exploratory approach to assess an expression and possible regulation of pro-angiogenic factors and tissue-degrading enzymes. Targeted gene expression profiling for pro-angiogenic factors under basal conditions revealed an expression of factors such as tumor necrosis factor (TNF)-alpha (37.4 SI), vascular endothelial growth factor (VEGF) B [signal

intensity (SI) 25.8 SI], interleukin (IL)-8 (SI 19.7), angiopoietin 1 (SI 6.4) or angiopoietin 2 (SI 7.2). After noxious stimulation with PMA, gene expression signal intensities increased for these factors, particularly in those with high baseline values such as TNF-alpha (SI 71.2) (Table 1).

Targeted expression profiling (Table 2) for tissue-degrading enzymes under basal, unstimulated conditions revealed an expression of different MMP, with higher intensities for MMP-14 (SI 10.2), MMP-2 (SI 11.3), and MMP-15 (SI 16.7) and lower intensities in MMPs such as MMP-7 (SI 3.3) and MMP-19 (SI 3.7). After noxious stimulation to mimic inflammatory conditions using a low concentration of PMA, SIs increased in these enzymes, with highest increases for MMP-14 (SI 13.7), MMP-2 (SI 14.3) and MMP-15 (SI 22.3).

Matrixmetalloproteinase expression

To verify data obtained from HMC-1 *matrixmetalloproteinase gene expression profiling*, MMP-2 expression was examined in normal skin and compared with atopic skin without and with mast cells located in the epidermis and in the papillary dermis (Fig. 3). Whereas epidermal MMP-2 expression was not found in normal skin and atopic dermatitis skin without epidermal mast cell localization, cellular staining was found in skin with epidermally located mast cells (Fig. 3).

Discussion

Angiogenesis represents an important physiological process implicated in integrated biological responses of the skin towards noxious stimuli (2, 21). In contrast to numerous reports on the role of angiogenesis in tumors (22), only little respect has been paid to its potential role in the pathogenesis of chronic inflammatory diseases such as atopic dermatitis. Angiogenesis is a multi-step event which is regulated by a large number of mediators. Many of them are known to be stored and released by mast cells (23), an inflammatory cell type which is known to play an important role in chronic inflammatory diseases such as atopic dermatitis (24).

As mast cell density within skin tissues correlates with the density of blood vessels (25), and activation of mast cells can be associated with the degradation of connective tissue, a participation of mast cells in neovascular processes underlying atopic dermatitis lesions may be suggested. As the potential significance of papillary and epidermal mast cells for cutaneous angiogenic mechanisms has not been investigated so far, the present study examined the presence of mast cells in the epidermis of atopic dermatitis lesions and their relation to vascular structures.

Out of 27 lesional biopsies, mast cells were found to be present in the epidermis of five biopsies from different

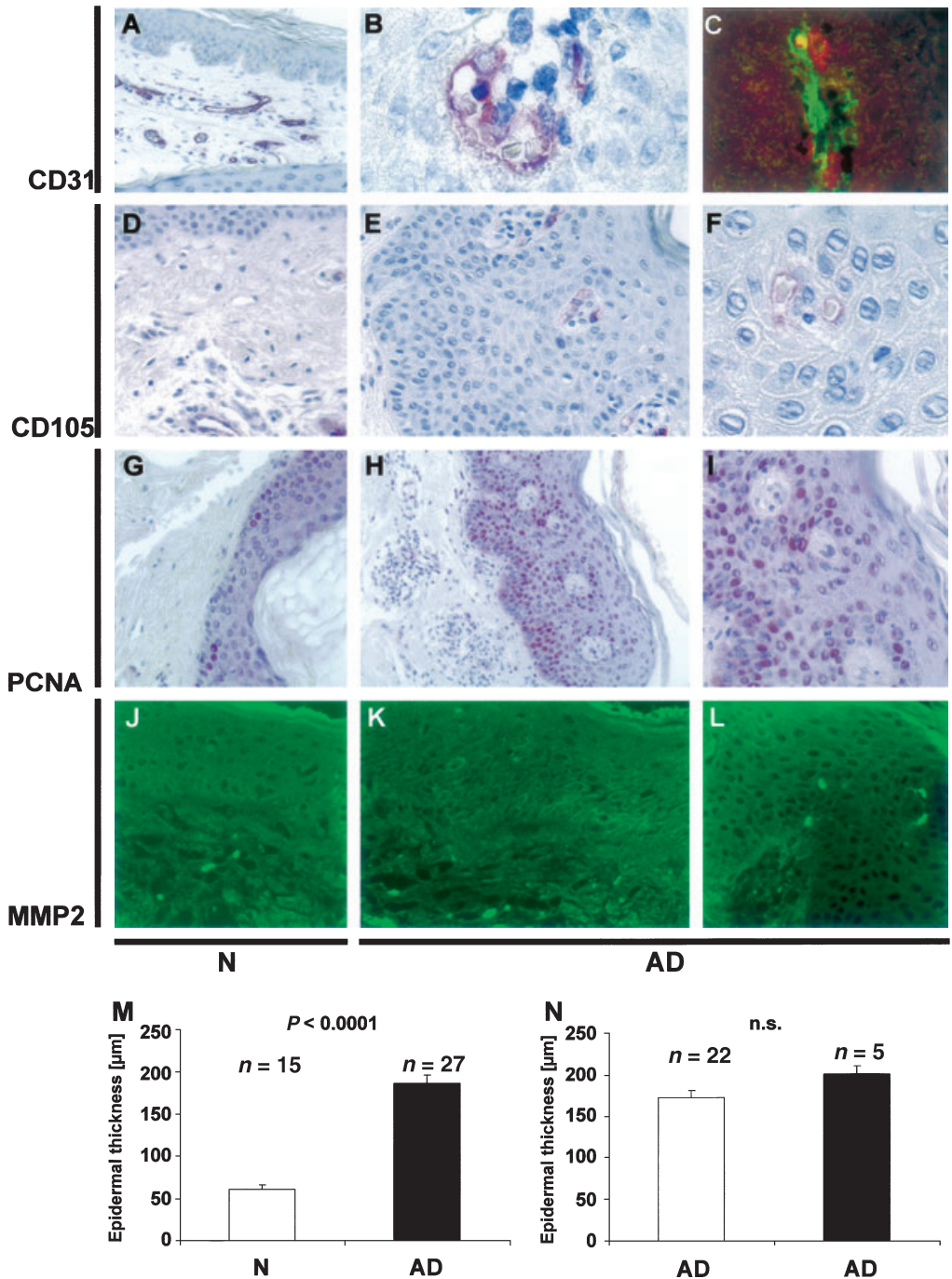


Figure 3. Relation to vascular structures and activity. Endothelial cell marker immunohistochemistry in normal skin (N) and atopic dermatitis skin (AD) was performed using antibodies specific against CD 31 [all endothelial cells, (A)–(C)] and CD 105 [only proliferating cells, (D)–(F)] and revealed a regular distribution in normal skin [(A) and (D)]. In atopic dermatitis, CD 31-positive cells (B) and CD 105-positive activated cells [(E) and (F)] were found in the epidermis. Double-labeling fluorescence immunohistochemistry for CD 31-positive endothelial cells (red) and c-kit-positive mast cells in atopic dermatitis demonstrated a juxtaposition of both cell types in the epidermis (C). Proliferating cell nuclear antigen (PCNA)-activity was increased in epidermal keratinocytes of atopic dermatitis biopsies [(H) and (I)] in comparison to normal skin (G). Expression of MMP-2 was examined in normal skin (J), atopic skin without epidermally located mast cells (K) and atopic skin with epidermally (L) located mast cells. Epidermal wall thickness was assessed by morphometry [(M) and (N)] with mean $\pm$ SEM epidermal thickness in normal skin compared to atopic dermatitis skin (J) and mean $\pm$ SEM epidermal thickness in atopic dermatitis with epidermal mast cells (ADw) compared to atopic dermatitis without mast cells in the epidermis (ADwo) (K). Original magnification $\times 100$ [(A), (D), (E), (G), (H) and (J)–(L)], $\times 200$ [(B), (C), (F) and (I)].

Table 1. Targeted gene expression profiling of pro-angiogenic factors

Gene name	Accession no.	Locus	Basal	Stimulated
Tumor necrosis factor- α	M10988	6p21.3	37.4	71.2
Vascular endothelial growth factor B	U43368	11q13	25.8	43.4
Interleukin-8	M17017	4q13-q21	19.7	30
Angiopoietin 2	NM_001146	8p23.1	7.2	12
Angiopoietin 1	NM_001146	8q23.1	6.4	9.7
PDGF	AV729452	7p21	5.3	7.2
EGF	X04571	4q25	4.7	6.0
Fibroblast activation protein (collagenase)	NM_004460	2q23	4.8	10.5

PDGF, platelet-derived growth factor; EGF, epidermal growth factor.

Table 2. Targeted gene expression profiling of tissue-degrading matrix metalloproteinase (MMP) enzymes

Gene name	Accession no.	Locus	Basal	Stimulated
MMP-15	NM_002428	16q13-16q21	16.7	22.3
MMP-2	J03210	16q13-q21	11.3	14.3
MMP-14	AW628280	14q11-q12	10.2	13.7
MMP-16	U79292	8q21	7.0	10.2
MMP-9	AX011001	20q11.2-q13.1	6.2	7.6
MMP-11	AI189375	22q11.23	5.3	9.2
MMP-1	AK024818	11q22.3	4.3	4.8
MMP-10	NM_002425	11q22.3	4.2	5.0
MMP-23B	AI347985	1p36.3	3.9	4.7
MMP-19	U38320	12q14	3.7	4.2
MMP-7	X07819	11q21-q22	3.3	4.3

patients. So far, only a preliminary study suggested the existence of this cell type in the epidermis and special fixation and staining techniques were reported to be required (7). Using a variety of detection systems, we were able to confirm these findings for five of the 27 patients. To identify the phenotype of papillary and epidermal mast cells, we examined their ability to express tryptase, chymase and c-kit and a differential expression of these mast cell markers was present in the population these mast cells.

Epidermal mast cells were not only found in close contact to the basal lamina but also reaching to the stratum granulosum, indicating a penetration to more superficial layers of the epidermis, which has not been shown in previous studies (7). Serial sections demonstrated an abundant staining for c-kit in epidermal and papillary mast cells. The expression of this stem cell factor (SCF) receptor indicates that papillary and epidermal mast cells can still be activated by SCF. The c-kit expression may serve as protection against pro-apoptotic factors. In this respect, similar to certain forms of mastocytosis, where a gain-of-function mutation in the c-kit gene was reported which prevents apoptosis (26), the expression of c-kit in epidermal and papillary mast cells may prevent apoptosis and thus triggering of inflammatory events.

As neovascular processes involve the action of angiogenic growth factors and require the localized proteolytic modification of the extracellular matrix (27), the presently

identified mast cells which express both tryptase and chymase may be implicated as accessory cells for cutaneous angiogenesis in lesions of atopic dermatitis: by having the capability to produce both mast-cell-specific serine proteases tryptase and chymase, as shown by the present immunohistochemical studies, epidermal and papillary mast cells may significantly contribute to proteolysis of matrix to facilitate neovascularization.

To characterize the anatomical relation of epidermal and papillary mast cells to surrounding vascular structures, endothelial markers were combined with mast cell markers. It was demonstrated that a large number of mast cells was in close proximity to endothelial cells. Fluorescence-double staining for mast cell marker c-kit and endothelial cell marker CD31 in the lesional atopic dermatitis epidermis confirmed this juxtaposition. These findings underline the potential of mast cells to function as active participants in cutaneous angiogenesis. In this respect, CD31-positive endothelial cells were only found to be sprouting from the papillae up to the stratum granulosum in the presence of surrounding mast cell which may facilitate the event of angiogenesis and neovascularization. With regard to the numerous reports which suggest an active participation of mast cells in the angiogenesis underlying neoplastic diseases (28, 29), the present study describes for the first time the potential of this inflammatory cell to facilitate angiogenesis in allergic diseases such as atopic dermatitis. The altered expression pattern of G protein-coupled receptors of vasoactive peptides in mast cells of atopic dermatitis patients underlines the potential role of mast cells in regulating angiogenesis in inflammatory cutaneous diseases (19, 30, 31).

To identify a possible relationship between angiogenesis and mast cell infiltration into the epidermis, the epidermal wall thickness was assessed. The maximal epidermal thickness which can be supplied with nutrients by diffusion of plasma from the dermis is about 150 μm . In the present study, the thickness of the normal epidermis was about 50 μm whereas the atopic dermatitis epidermis was threefold thicker. However, there was no significant difference in thickness between lesions with epidermal mast cells and lesions without epidermal mast

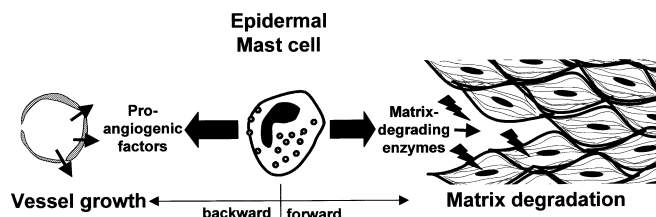


Figure 4. ‘Tunnel’ hypothesis of bidirectional mast cell orchestration of angiogenesis in the epidermis. Epidermal and papillary mast cells propagate angiogenesis by forming a tunnel for new vessels. ‘Backward’ secretion of pro-angiogenic factors leads to stimulation of vessel sprouting and ‘forward’ secretion of matrix-degrading enzymes such as MMPs leads to the formation of an epidermal and papillary ‘tunnel’ for vessel growth.

cells. It is thus unlikely that mast cells are the only stimulus for the induction of angiogenesis in atopic dermatitis. In fact, epidermal keratinocytes are other likely contributors, also in view of their ability to produce VEGF and other pro-angiogenic factors (23, 32).

Based on the present findings of abundant mast cells in the epidermis and in view of the mast cell phenotype in this location, suggesting high cellular activity, the following hypothesis may be advanced: mast cells may function as triggers for vessel growth towards the inflamed epidermis. To orchestrate these pathophysiological mechanisms, epidermally and papillary located mast cells may secrete factors propagating angiogenesis in a ‘forward’ and ‘backward’ manner (Fig. 4). In the backward direction inducing sprouting of vessels, release of pro-angiogenic factors would be expected whereas in the forward direction, secretion of tissue-degrading enzymes such as MMPs would allow for the advancement of vessels into the tissue. In order to test this hypothesis, we performed differential gene expression profiles for pro-angiogenic factors and for tissue-degrading enzymes in the human mast cell line 1 under basal conditions and under mild noxious stimulation to mimic an inflammatory situation. The studies revealed an increased expression of several potent pro-angiogenic

factors which may function as backward stimuli for vascular growth after stimulation. Also, stimulation led to an increase of MMP expression which would be secreted by mast cells in order to degrade the epidermal matrix to build a preformed ‘tunnel’ for new vessels. Expression of MMP-2 was also demonstrated by immunohistochemistry in atopic dermatitis skin with epidermally located mast cells supporting this theory.

With regard to the inflammatory processes associated with atopic dermatitis, an increased proliferative activity of the lesional epidermis can be assumed. To characterize this activity which may lead to the stimulation of vessel growth due to an enhanced nutritional need or increased hypoxia (32), immunohistochemistry for PCNA was carried out. An abundant signaling was not only found in cells of the basal layers, but also in keratinocytes of more superficial layers. In contrast to a previous study which localized PCNA-activity in mild to moderate and in severe atopic dermatitis only to basal layers (33), PCNA-positive cells were presently found in superficial layers as well, possibly due to more severe disease in the patients studied here.

In summary, mast cells are able to migrate towards the epidermis of atopic dermatitis skin lesions. Here and in papillary areas, they may stimulate neoangiogenesis via the release of proangiogenic factors and help to facilitate vessel growth by secreting different proteases such as MMPs. Via the new vessels, inflammatory cells may then easily migrate to the epidermal layer, plasma components may more easily diffuse into the epidermis, and both may maintain the processes of chronic inflammation as well as aid in the defense against noxious stimuli and allergens.

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