

Pimecrolimus Induces Apoptosis of Mast Cells in a Murine Model of Cutaneous Mastocytosis

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Key Words

Apoptosis · Cutaneous mastocytosis · Histamine · Mast cell · Murine model · Pimecrolimus · Stem cell factor

Abstract

Background: Cutaneous mastocytosis (CM) is a common type of mastocytosis. Current treatment of CM is generally symptomatic. Pimecrolimus has been demonstrated as an effective anti-inflammatory drug for the treatment of inflammatory skin diseases, but whether it treats CM remains unknown. **Methods:** The murine model of CM was induced by subcutaneous injection of 100 µg/kg recombinant murine stem cell factor (rmSCF) for a total of 17 days in Balb/c mice. Beginning on the 8th day, treatment with pimecrolimus 1% cream or vehicle was performed topically and daily for 10 days. The clinical signs of CM were scored, and pathological analysis was performed with toluidine blue staining and hematoxylin and eosin staining. The in situ apoptotic mast cells (MCs) were studied by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. The cutaneous histamine level was measured by ELISA. **Results:** In the rmSCF-treated mice, the clinical signs of CM, including erythema, wheal after rubbing lesion skins, and increased thickness of skin, were obvious compared to control mice, and were reduced after pimecrolimus treatment. The numbers of cutaneous MCs and neutrophils were significantly greater in mice with CM than in control mice, and pimecrolimus

treatment decreased the numbers of MCs but not neutrophils. Extensive apoptosis of cutaneous MCs was observed in pimecrolimus-treated mice. The cutaneous histamine level was elevated in the mice with CM compared with healthy controls, and was lowered after treatment with pimecrolimus. **Conclusions:** Pimecrolimus effectively treats CM by reducing the density of cutaneous MCs and the subsequent histamine production through inducing MCs apoptosis.

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Background

Mastocytosis is a disease characterized by increased numbers of mast cells (MCs) in various organs, particularly in cutaneous tissue, which is termed cutaneous mastocytosis (CM) [1]. It presents as red-brown macules, papules or plaques on the skin that often develop wheals, erythema and pruritus after stroking or rubbing the lesions, i.e. Darier's sign. MCs secrete multiple mediators, including histamine, heparin, serine proteases, lipid-derived mediators, and cytokines, which cause the clinical signs of CM [2]. Stem cell factor (SCF) is the principal human MC growth factor. It binds to its receptor, KIT, and stim-

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ulates the differentiation and proliferation of MCs from CD34⁺ cells. It also promotes chemotaxis, adhesion, activation, and the survival of MCs by inhibition of apoptosis [3]. Longley et al. [4] showed that SCF levels in the dermis and epidermal keratinocytes were significantly elevated in patients with CM. Increased expression of SCF was also found in patients with indolent systemic mastocytosis [5]. Activation mutations of *c-kit* lead to constitutive autophosphorylation of the SCF receptor KIT and, in turn, the uncontrolled growth and survival of MC and other SCF-mediated physiological functions. Such mutations of KIT have been demonstrated in patients with mastocytosis, including CM, and have been considered as the best-recognized molecular marker of mastocytosis [6]. These results indicate that overactivation of the SCF/KIT pathway by either overexpression of SCF or activation mutations of KIT plays an important role in the pathogenesis of mastocytosis [2].

The current treatment of CM is mainly symptomatic and directed towards the skin lesions and systemic signs caused by the release of MC mediators but does not change the underlying cause of the disease: aberrant numbers of MCs. Mainstay treatments include avoidance of trigger factors, application of agents, such as MC stabilizers, H1 and H2 receptor antagonists, corticosteroids, and psoralen-UVA photochemotherapy [7]. Since the SCF/KIT pathway is the principle regulator of MCs, inhibition of this pathway should theoretically be effective in the treatment of mastocytosis. Recently, several tyrosine kinase inhibitors that inhibit the kinase activity of KIT have been shown to inhibit proliferation and mediator release of neoplastic MCs in vitro and to have a positive clinical response in patients with mastocytosis [8–10].

Pimecrolimus (SDZ ASM 981) is the 33-epichloro-derivative of the macrolactam ascomycin and a specific calcineurin inhibitor [reviewed in references 11, 12]. Its specific target cells include T cells and MCs. Pimecrolimus inhibits T cell proliferation, activation and release of growth factors and pro-inflammatory mediators. It also blocks the release of mediators from MCs. Pimecrolimus combines high anti-inflammatory activity in skin with a low potential to impair systemic immune reactions, which makes it the first alternative to topical corticosteroids for the treatment of a number of inflammatory skin diseases, including atopic dermatitis, allergic contact dermatitis, irritant contact dermatitis and vitiligo.

In the present work, we tested the effects of pimecrolimus on a murine model of CM and found that it effectively treats CM by inducing cutaneous MC apoptosis in vivo.

Methods

Induction of a Murine CM Model and Treatment with Pimecrolimus

The murine CM model was induced in female Balb/c mice (6–8 weeks of age; The Jackson Laboratory) by s.c. injection of recombinant murine SCF (rmSCF) according to the protocol described before with modifications [13]. Three groups were included: healthy control group; rmSCF group, and rmSCF with pimecrolimus treatment group (10 mice were used for each group). Briefly, mice received daily s.c. injection of rmSCF (Peprotech) at 100 µg/kg for a total of 17 days. The control group received saline vehicle. Starting on the 8th day, mice receiving rmSCF were treated daily with topical pimecrolimus 1% cream (Elidel®) or vehicle (Novartis) close to the injection sites for 10 days. Twenty-four hours after the last treatment with pimecrolimus, the skin tissues surrounding the injection sites were cut off for pathological study and the measurement of histamine levels. All animal use procedures were approved by the Los Angeles Biomedical Research Institute Animal Care and Use Committee at Harbor-UCLA Medical Center.

Quantification of Clinical Signs of CM

The clinical signs of CM of each mouse, including erythema, wheals after rubbing skin, and inflammation (thickness of skin fold), were assessed daily by a blinded investigator. The severity of CM was quantified by measuring severity of erythema, wheals and thickness of skin (0–2 for each category), and it was respectively graded by adding numbers 0–2 from each category. The least severe skin condition was recorded as '0' and the most severe skin condition was recorded as '6'. The assessment of clinical signs in each mouse was performed 24 h after the last treatment with pimecrolimus.

Skin Pathological Analysis

To reveal cutaneous MCs and inflammatory cells, toluidine blue and H&E staining was performed, respectively. The numbers of MCs, neutrophils and eosinophils were obtained by the independent observer.

In situ Apoptosis by TUNEL Assay

To study in situ apoptosis of cutaneous MCs, the skin samples were studied with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using the ApopTag Kit (Oncor). The slides were counterstained with methyl green. The apoptotic MCs were counted as described above.

Measurement of Histamine Levels in Skin

The frozen skin tissue was smashed, sonicated and centrifuged at 10,000 g and 4°C for 30 min. The amount of total proteins in the supernatant was determined using DC Protein Assay Kit (Bio-Rad). The histamine levels in the supernatant were measured using Histamine EIA Kit (Cayman Chemical). The final results were expressed by the amount of histamine per milligram total skin proteins.

Statistic Analysis

The results were reported as the means ± SE of the combined experiments. Differences were analyzed for significance by means of ANOVA. A p value <0.05 was considered statistically significant.

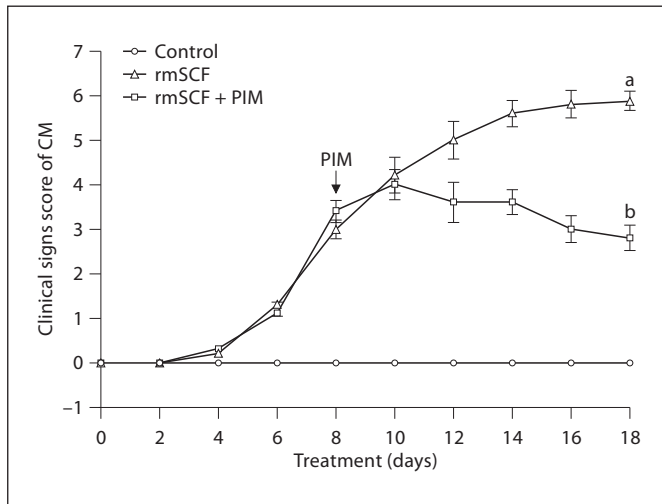


Fig. 1. Effects of pimecrolimus on clinical signs of CM in the murine CM model. The severity of CM was scored in control mice, mice receiving rmSCF alone or rmSCF and pimecrolimus (PIM). Values were shown as mean $\pm$ SE ($n = 10$ mice). ^a $p < 0.00001$ vs. control group; ^b $p < 0.0001$ vs. rmSCF group.

Results

After 6 days of s.c. administration of rmSCF, the clinical signs of CM began to appear, including mild erythema, positive wheal after rubbing skin close to the injection sites, and increased inflammation evident by the elevated thickness of skin. At the end of the study (after 17 days of rmSCF injection) the clinical signs of CM were evident, whereas control mice showed no signs of CM ($p < 0.00001$). Pimecrolimus treatment reduced the severity of CM by 51.7% compared with mice treated with rmSCF alone ($p < 0.0001$; fig. 1).

The skin of rmSCF-treated mice showed extensive MC infiltrates of the papillary and reticular dermis. Cutaneous MC numbers in mice receiving rmSCF alone were 5.2-fold higher than in control mice (42.8 ± 5.1 vs. 8.2 ± 1.3 per mm^2 , $p < 0.001$). Pimecrolimus treatment reduced the number of cutaneous MCs by 58% compared to mice receiving rmSCF alone (18 ± 3.6 vs. 42.8 ± 5.1 per mm^2 , $p < 0.01$; fig. 2).

The inflammatory cells infiltrating the dermis were predominantly neutrophils. The number of neutrophils in rmSCF-treated mice was 3.2-fold higher than in control mice (34.7 ± 9.7 vs. 11.0 ± 1.1 per mm^2 , $p < 0.01$). Pimecrolimus treatment had no significant effects on the numbers of cutaneous neutrophils (26.4 ± 5.4 vs. 34.7 ± 9.7 per mm^2 , $p = 0.29$; fig. 3). The infiltration of eosino-

phils into the dermis was not affected by either rmSCF injection or pimecrolimus treatment (fig. 3 and data not shown).

The number of apoptotic MCs in the pimecrolimus-treated mice was 8-fold and 10.5-fold greater than in control mice and mice receiving rmSCF alone, respectively [9.6 ± 1.1 vs. 1.2 ± 0.3 (control group) or 0.92 ± 0.2 (rmSCF group) per mm^2 , $p < 0.001$; fig. 4]. The percentage of apoptotic cutaneous MCs in rmSCF-treated mice was the lowest ($2.2 \pm 0.08\%$), and it was 6.6-fold higher in healthy control mice ($14.6 \pm 0.4\%$, $p < 0.01$ vs. rmSCF group), whereas it was the highest in rmSCF-treated mice receiving pimecrolimus treatment ($53.3 \pm 7.6\%$, $p < 0.01$ vs. control or rmSCF-treated mice).

Finally, the level of skin histamine of mice receiving rmSCF was 28.5 times higher than in control mice (779.6 ± 60.3 vs. 27.4 ± 3.4 nmol/mg proteins, $p < 0.0001$). After pimecrolimus treatment, it was reduced by 78.5% compared with mice treated with rmSCF alone (168.1 ± 33.2 vs. 779.6 ± 60.3 nmol/mg proteins, $p < 0.001$).

Discussion

Costa et al. [14] reported that s.c. injection of human SCF in patients induced obvious wheals with surrounding erythema, flare reaction, hyperpigmentation and hyperplasia of cutaneous MCs close to the injection sites. SCF also promoted degranulation of the overproliferated MCs, which was indicated by elevated urinary levels of the histamine metabolite and serum levels of MC α -tryptase. Similar results were observed in another clinical study [15]. All or some clinical signs of CM described above were also observed in mice, rat, dog or nonhuman primates that received s.c. injection of SCF [13, 16–18]. These results indicate that s.c. injections of SCF can induce the main clinical signs of CM in skin close to the injection sites, and are useful to establish an animal CM model. In our murine CM model, the main clinical signs of CM were observed, including Darier's sign, cutaneous MC hyperplasia and accumulated MC mediators (such as histamine) in skin. After pimecrolimus treatment, the severity of these CM signs was reduced, suggesting that pimecrolimus effectively treats CM.

Mastocytosis represents a generalized overproliferation and accumulation of MCs. It is reasonable to speculate that there is an imbalance of MC proliferation and elimination in mastocytosis lesions. Hartmann et al. [19] reported that in patients with cutaneous mastocytosis the cutaneous MC accumulation was associated with a de-

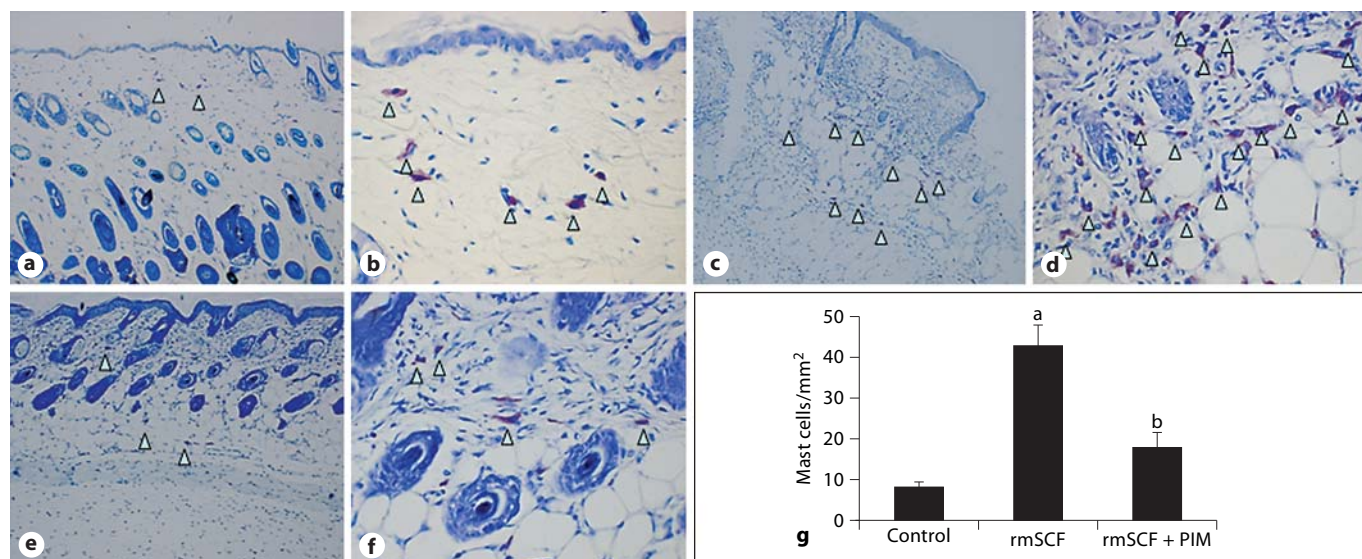


Fig. 2. Effects of pimecrolimus on the density of cutaneous MCs in the murine CM model by toluidine blue staining. **a, b** Control group. **c, d** rmSCF group. **e, f** rmSCF + pimecrolimus group. Original magnification: $\times 10$ (**a, c, e**), $\times 40$ (**b, d, f**). MCs residing in the dermis with pink metachromatic cytoplasm are indicated by arrowheads. **g** MC numbers were counted and are expressed as mean $\pm$ SE per mm² (n = 10 mice). PIM = Pimecrolimus. ^a $p < 0.001$ vs. control group; ^b $p < 0.01$ vs. rmSCF group.

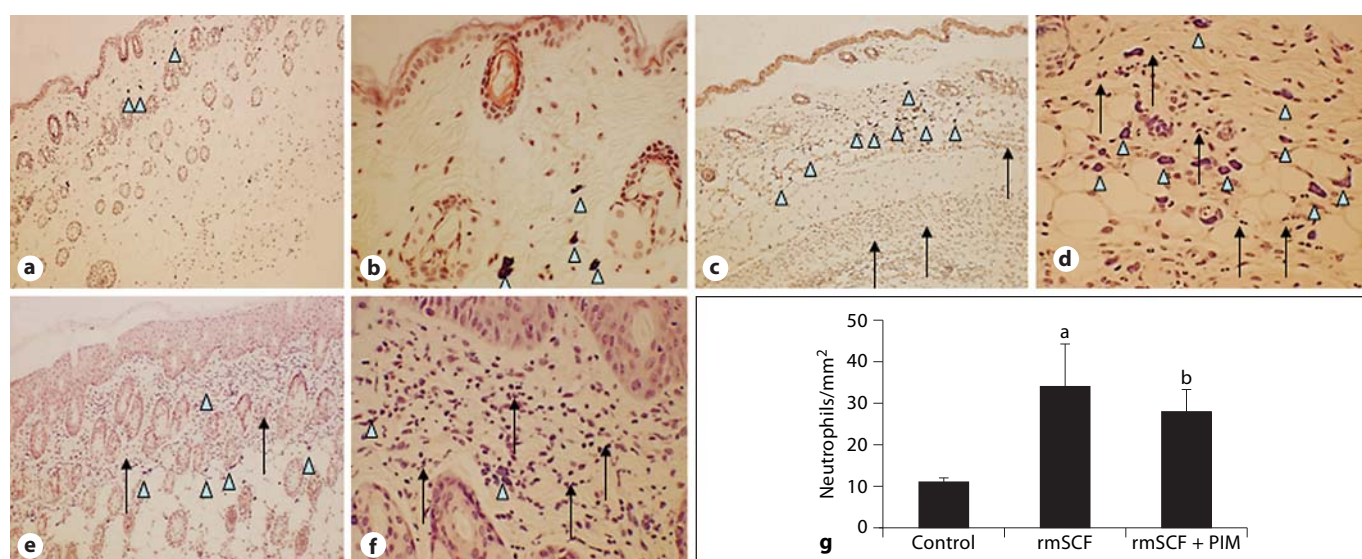


Fig. 3. Effects of pimecrolimus on cutaneous inflammation in the murine CM model by H&E staining. **a, b** Control group. **c, d** rmSCF group. **e, f** rmSCF + pimecrolimus group. Original magnification: $\times 10$ (**a, c, e**), $\times 40$ (**b, d, f**). Neutrophils and MCs are indicated by arrows and arrowheads, respectively. **g** Neutrophil numbers are expressed as mean $\pm$ SE per mm² (n = 10 mice). PIM = Pimecrolimus. ^a $p < 0.01$ vs. control group; ^b $p = 0.29$ vs. rmSCF group.

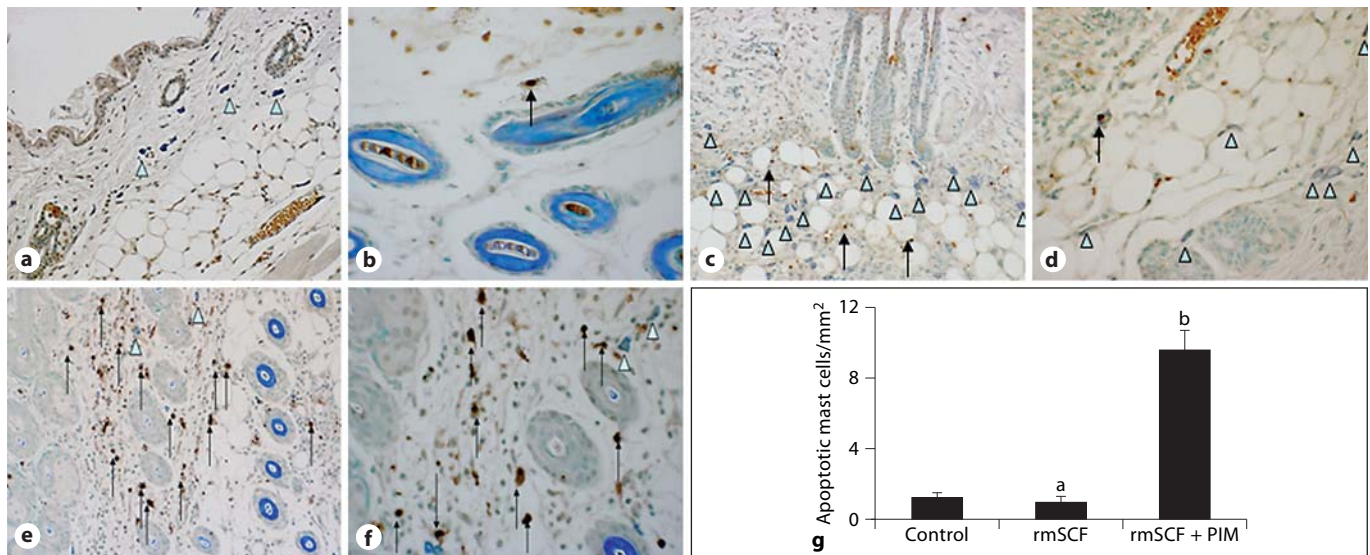


Fig. 4. Effects of pimecrolimus on apoptosis of cutaneous MCs in the murine CM model by TUNEL assay. **a, b** Control group. **c, d** rmSCF group. **e, f** rmSCF + pimecrolimus group. Original magnification: $\times 20$ (**a, c, e**), $\times 40$ (**b, d, f**). Arrowheads show MCs not undergoing apoptosis and arrows show apoptotic MCs. **g** Numbers of apoptotic MCs are expressed as mean $\pm$ SE per mm² (n = 10 mice). PIM = Pimecrolimus. ^a p = 0.19 vs. control group; ^b p < 0.001 vs. control or rmSCF group.

creased rate of apoptosis of MCs. Bcl-2 protein is a strong inhibitor of apoptosis. In cutaneous MCs from patients with mastocytosis, Bcl-2 expression is strongly enhanced compared to healthy controls [20]. These results suggest that altered MC proliferation and apoptosis contribute to the pathogenesis of mastocytosis. In our study, after 17 days of s.c. injection of rmSCF, the MC density in skin was significantly increased compared with control mice; however, the percentage of apoptotic MCs was only 2.2% whereas it was as high as 14.6% in control mice. After pimecrolimus treatment, the density of cutaneous MCs was significantly reduced, whereas the number of neutrophils was not affected, despite the hyperplasia of both types of cells after SCF injection. This suggests that pimecrolimus appears to selectively inhibit SCF-induced accumulation of cutaneous MCs but not neutrophils.

The possible mechanisms for the accumulation of cutaneous MCs caused by s.c. SCF administration include stimulating proliferation of cutaneous MCs or MC progenitor cells resident in the skin or immigrating from the blood, enhancing immigration of MC progenitor cells from the blood to skin, or reducing the rate of apoptosis of MCs, and pimecrolimus may inhibit SCF-induced accumulation of MCs through disturbing one or more of these mechanisms. For the first time, we found that

pimecrolimus caused extensive apoptosis of cutaneous MCs but not neutrophils. The percentage of apoptotic cutaneous MCs after pimecrolimus treatment in the mice with CM was significantly higher than in healthy control mice or mice receiving SCF alone. Pimecrolimus has been shown to induce T cell apoptosis [21]. Our results indicate that pimecrolimus is also capable of inducing apoptosis of cutaneous MCs in vivo. Moreover, we also observed that pimecrolimus shows no obvious effects on cutaneous MCs in the healthy mice not receiving rmSCF but only saline injection (data not shown), suggesting that pimecrolimus may specifically induce apoptosis of MCs under proliferation status but not resting status. However, whether pimecrolimus directly inhibits the proliferation and immigration of MCs and MC progenitors needs to be verified in the future. Further studies to identify the involved molecular mechanisms for pimecrolimus-mediated reduction of accumulation of cutaneous MCs would promote our understanding of the mechanisms by which pimecrolimus treats CM.

Finally, we found that the skin histamine levels were significantly elevated in rmSCF-treated mice compared with normal mice, which suggests that in our murine CM model, the overproliferated MCs were functionally activated. Similar results were observed in patients, rats and

mice receiving s.c. injection of SCF [14, 16, 17]. After treatment with pimecrolimus, the histamine levels were reduced. Pimecrolimus has been shown to inhibit the release of mediators from MCs [22]. The inhibitory effect on histamine levels observed in our study may be the combined inhibitory effects of pimecrolimus on both the MC density and MC degranulation. In summary, for the first time we found that pimecrolimus effectively treats CM in a murine model through reducing the underlying lesion, aberrant numbers of cutaneous MCs, and subsequent elevated levels of histamine in the skin lesions of CM. We further showed that the mechanism for pimecrolimus-mediated reduction of cutaneous MCs is the induction of apoptosis. Topical application of pimecrolim-

us has been used to successfully treat the patient with telangiectasia macularis eruptiva perstans, a form of CM [23]. This report together with our findings suggests that pimecrolimus may be a new effective candidate for the treatment of CM and possibly other mastocytosis diseases.

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References

- Horny HP, Sotlar K, Valent P: Mastocytosis: state of the art. *Pathobiology* 2007;74:121–132.
- Metcalfe DD: Mast cells and mastocytosis. *Blood* 2008;112:946–956.
- Reber L, Da Silva CA, Frossard N: Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. *Eur J Pharmacol* 2006; 533:327–340.
- Longley BJ Jr, Morganroth GS, Tyrrell L, Ding TG, Anderson DM, Williams DE, Halaban R: Altered metabolism of mast-cell growth factor (c-kit ligand) in cutaneous mastocytosis. *N Engl J Med* 1993;328:1302–1307.
- Akin C, Jaffe ES, Raffeld M, Kirshenbaum AS, Daley T, Noel P, Metcalfe DD: An immunohistochemical study of the bone marrow lesions of systemic mastocytosis: expression of stem cell factor by lesional MCs. *Am J Clin Pathol* 2002;118:242–247.
- Lim KH, Pardanani A, Tefferi A: KIT and mastocytosis. *Acta Haematol* 2008;119:194–198.
- Briley LD, Phillips CM: Cutaneous mastocytosis: a review focusing on the pediatric population. *Clin Pediatr (Phila)* 2008;47:757–761.
- Krauth MT, Mirkina I, Herrmann H, Baumgartner C, Kneidinger M, Valent P: Midostaurin (PKC412) inhibits immunoglobulin E-dependent activation and mediator release in human blood basophils and mast cells. *Clin Exp Allergy* 2009;39:1711–1720.
- Akin C, Fumo G, Yavuz AS, Lipsky PE, Neckers L, Metcalfe DD: A novel form of mastocytosis associated with a transmembrane c-kit mutation and response to imatinib. *Blood* 2004;103:3222–3225.
- Patnaik MM, Tefferi A, Pardanani A: Kit: molecule of interest for the diagnosis and treatment of mastocytosis and other neoplastic disorders. *Curr Cancer Drug Targets* 2007;7:492–503.
- Stuetz A, Baumann K, Grassberger M, Wolff K, Meingassner JG: Discovery of topical calcineurin inhibitors and pharmacological profile of pimecrolimus. *Int Arch Allergy Immunol* 2006;141:199–212.
- Day I, Lin AN: Use of pimecrolimus cream in disorders other than atopic dermatitis. *J Cutan Med Surg* 2008;12:17–26.
- Tsai M, Shih LS, Newlands GF, Takeishi T, Langley KE, Zsebo KM, Miller HRP, Geissler EN, Galli SJ: The rat c-kit ligand, stem cell factor, induces the development of connective tissue-type and mucosal MCs in vivo: analysis by anatomical distribution, histochemistry, and protease phenotype. *J Exp Med* 1991;174:125–131.
- Costa JJ, Demetri GD, Harrit TJ, Dvorak AM, Hayes DF, Merica EA, Menchaca DM, Gringeri AJ, Schwartz LB, Galli SJ: Recombinant human stem cell factor (kit ligand) promotes human MC and melanocyte hyperplasia and functional activation in vivo. *J Exp Med* 1996;183:2681–2686.
- Dvorak AM, Costa JJ, Morgan ES, Monahan-Earley RA, Galli SJ: Diamine oxidase-gold ultrastructural localization of histamine in human skin biopsies containing MCs stimulated to degranulate in vivo by exposure to recombinant human stem cell factor. *Blood* 1997;90:2893–2900.
- Galli SJ, Iemura A, Garlick DS, Gamba-Vitalo C, Zsebo KM, Andrews RG: Reversible expansion of primate MC populations in vivo by stem cell factor. *J Clin Invest* 1993;91: 148–152.
- Maurer M, Galli SJ: Lack of significant skin inflammation during elimination by apoptosis of large numbers of mouse cutaneous MCs after cessation of treatment with stem cell factor. *Lab Invest* 2004;84:1593–1602.
- Hammerberg B, Olivry T, Orton SM: Skin MC histamine release following stem cell factor and high-affinity immunoglobulin E receptor cross-linking in dogs with atopic dermatitis. *Vet Dermatol* 2001;12:339–346.
- Hartmann K, Hermes B, Rappersberger K, Sepp N, Mekori YA, Henz BM: Evidence for altered MC proliferation and apoptosis in cutaneous mastocytosis. *Br J Dermatol* 2003; 149:554–559.
- Hartmann K, Artuc M, Baldus SE, Zirbes TK, Hermes B, Thiele J, Mekori YA, Henz BM: Expression of Bcl-2 and Bcl-xL in cutaneous and bone marrow lesions of mastocytosis. *Am J Pathol* 2003;163:819–826.
- Hoetzenecker W, Ecker R, Kopp T, Stuetz A, Stingl G, Elbe-Bürger A: Pimecrolimus leads to an apoptosis-induced depletion of T cells but not Langerhans cells in patients with atopic dermatitis. *J Allergy Clin Immunol* 2005;115:1276–1283.
- Zuberbier T, Chong SU, Grunow K, Guhl S, Welker P, Grassberger M, Henz BM: The ascomycin macrolactam pimecrolimus (Elidel, SDZ ASM 981) is a potent inhibitor of mediator release from human dermal MCs and peripheral blood basophils. *J Allergy Clin Immunol* 2001;108:275–280.
- Lee HW, Jeong YI, Choi JC, Lee MW, Choi JH, Moon KC, Koh JK: Two cases of telangiectasia macularis eruptiva perstans demonstrated by immunohistochemistry for c-kit (CD 117). *J Dermatol* 2005;32:817–820.