

Original article

Association of stem cell factor expression in nasal polyp epithelial cells with aspirin sensitivity and asthma

Mast cells constitute a significant proportion of cells infiltrating nasal polyp tissue, and epithelial cells may release stem cell factor (SCF), a cytokine with chemotactic and survival activity for mast cells. We aimed to assess the expression of SCF in human nasal polyp epithelial cells (NPECs) as related to patients' clinical phenotypes. Nasal polyp tissues were obtained from 29 patients [including nine with aspirin (ASA)-hypersensitivity and 12 with bronchial asthma] undergoing polypectomy for nasal obstruction. Epithelial cells were obtained following 6-week culture of nasal polyps explants. The SCF released into the culture supernatant was assessed by enzyme-linked immunosorbent assay (ELISA) and total SCF mRNA in the polyp tissue was determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). For the whole group of patients, the number of polypectomies correlated with expression of SCF mRNA ($r = 0.62$; $P < 0.005$), SCF protein in the NPECs supernatants ($r = 0.39$; $P < 0.05$) and with density of mast cells in epithelial layer ($r = 0.37$; $P < 0.05$) and stromal layer ($r = 0.5$; $P < 0.01$) of nasal polyps. The SCF/ β -actin mRNA ratios were significantly higher in ASA-hypersensitive (AH) asthmatics (median 0.97, range: 0.8–1.5) when compared with ASA-tolerant (AT) patients (median 0.5, range: 0.1–0.7; $P < 0.001$). The SCF protein concentration in NPEC supernatants was also significantly higher in AH asthmatics (median 1.10 pg/ μ g DNA, range: 0.4–1.9) when compared with AT patients (median 0.1 pg/ μ g DNA, range: 0.02–1.2; $P < 0.001$). In the subpopulation of ASA-sensitive asthmatics the number of polypectomies correlated also with the density of mast cells and eosinophils in the polyp tissue.

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Nasal polyps develop as a manifestation of chronic upper airway inflammation, and involve proliferation of fibroblasts, deposition of connective tissue, glandular hypertrophy, and inflammatory cell infiltration into these lesions (1–4). Eosinophils and mast cells comprise the most abundant populations of cells infiltrating nasal polyps and by released mediators and cytokines seem to contribute to the pathogenesis of the disease (5–9). The mechanism of mast cells recruitment and survival in nasal polyps is not known and does not seem to be related to allergy since similar density of mast cells can be found in both atopic and nonatopic polyps (10). Mast cells are more abundant in the superficial layer than in stromal layer (SL) of the polyp mucosa suggesting that epithelium may be a source of chemotactic and survival factors for mast cells. A growth factor potentially responsible for mast cells proliferation in nasal polyps may be stem cell factor (SCF). The SCF also known as c-Kit ligand is a pleiotropic cytokine involved in

the development and function of mast cells. The SCF has been identified as growth, differentiation, chemotactic and activation factor for mast cells in humans (11–14). Stem cell factor had been previously identified in the nasal epithelium from biopsy specimens and expression of SCF in cultured nasal epithelial cells was determined (15, 16).

The most severe form of nasal polyposis with high polyp recurrency rate is associated with aspirin (ASA) hypersensitivity and bronchial asthma (17). Nasal polyps from ASA-hypersensitive (AH) patients have a distinct profile of mediators and infiltrating cells with eosinophils being definitely the most prominent cell type in the polyp tissue (18–20). The second most abundant inflammatory cell in nasal polyps is mast cell, which is often found in the subepithelial layer, within airway epithelium or on the surface of the mucosa (10, 19). We hypothesized, which epithelial cells by release of SCF, a known mast cells growth factor may contribute to the pathogenesis of the inflammatory infiltrate in the nasal polyp and indirectly to the clinical severity of the disease. Although the expression of SCF in epithelial cells cultured from nasal mucosa and polyposis has been previously demonstrated no study

Abbreviations: SCF, stem cell factor; AH, aspirin-hypersensitive; AT, aspirin-tolerant; NPEC, nasal polyp epithelial cells; ESL, epithelial and subepithelial layer; SL, stromal layer.

attempted to relate SCF expression to clinical phenotypes or histopathology of individual polyps (15, 21).

Material and methods

Patients

Nasal polyps were obtained from 29 patients with chronic rhinosinusitis undergoing elective nasal surgery for reasons unrelated to the goals of this study. Rhinosinusitis was diagnosed based on clinical history of recurrent symptoms and presence of nasal polyps was confirmed by computerized tomography (CT) scans. Twelve patients suffered from bronchial asthma and nine of them were sensitive to ASA and other nonsteroidal anti-inflammatory drugs (NSAIDs). Bronchial asthma was diagnosed based on GINA2 Guidelines Criteria. Positive history of bronchial and/or nasal reaction to ASA or other NSAIDs was confirmed by positive nasal or inhalation challenge with lysine ASA as previously described (22). Atopy was defined by positive personal history of allergic respiratory symptoms and positive skin prick tests (SPT; wheal > 3 mm) to the panel of inhalant allergens (including *Dermatophagoides pteronyssinus*, *D. farinae*, mixed grass pollen, mixed tree pollen, cat and dog epithelium and *Alternaria alternata*). Nonatopic subjects had negative allergic history and negative SPT to the same panel of inhalant allergens. None of the patients had received oral or intranasal steroids for at least 4 weeks before surgery. The clinical characteristic of the subjects are summarized in Table 1.

The study was approved by local medical ethics committee and all subjects gave an informed consent.

Nasal polypectomy and tissue handling

Nasal polypectomies were performed under local anesthesia. Prior to surgery, 2% tetracaine HCl and 0.25% phenylephrine HCl were applied topically to the turbinates, nasal septum and middle meatus. After decongesting, the polyp was injected with 2–4 ml of 1% lidocaine with 1 : 100 000 adrenaline. Then, the polyp was removed using gentle traction and snare technique. Polyps were placed in ice-cold Medium 199 (Sigma, St Louis, MO, USA) and immediately transported to the laboratory in a thermoinsulation container (temperature 4–6°C).

Epithelial cell culture. The NPEC were cultured by technique of nasal polyps explants, as described by Devalia et al. (23). Briefly, after preserving a fragment of polyp tissue for histopathological investigations, the nasal polyp tissue was dissected into pieces approximately 2 mm³ in size and five or six sections-explants were transferred to 6 cm diameter Falcon ‘Primaria’ culture dishes

(Becton Dickinson Ltd, Oxford, UK). The explants were incubated, at 37°C in 5% CO₂ in air atmosphere in 2 ml of a complete culture medium. Prior to use, the medium was prepared in 100 ml quantities and contained 2.5 ml Nu-Serum IV (BD Biosciences, Bedford, MA, USA), 250 µg bovine pancreatic insulin, 250 µg human transferrin, 36 µg of hydrocortisone, 3 mg L-glutamine, 1.5 µg epithelial growth factor (EGF), penicillin (10 000 IU), streptomycin (10 mg) and amphotericin (250 µg) (Sigma) in M-199 (Sigma). The NPEC were obtained following 5 or 6 weeks culture. Culture media were changed every 48 h until confluence was reached, than the cells were cultured for 24 h in serum-free medium prior to experimental procedure. Finally, supernatants were removed for the SCF protein measurement, an aliquot of cells was took for immunocytochemistry and the remaining cells were lysed for RNA isolation.

Cytospine cell preparations from each culture were stained (peroxidase-antiperoxidase technology) using primary monoclonal antibody against human cytokeratin antigen (anti-CK-1; Dako, Copenhagen, Denmark) (22). Positive staining for cytokeratin and the morphological characteristics indicated that more than 96% (median: 96.5%, range 87–99) of cells were epithelial cells.

Detection of SCF with RT-PCR

Total RNA was extracted from cultured cells using the acid guanidinium thiocyanate-phenol-chloroform method (24) and reverse transcriptase polymerase chain reaction (RT-PCR) procedure was used as described previously (14). Briefly, 2 µg of total RNA was used for the synthesis of first stand cDNA with 200 U Moloney murine leukaemia virus (MMLV) reverse transcriptase and 0.5 µg oligo dT primer. Aliquots of each 5 µl cDNA were amplified to the appropriate cycle with 10 µM of a set of oligonucleotide primers, 25 nM/l of dNTPs and 1 U of *Taq* polymerase in 20 µl of reaction mixture. A set of oligonucleotide primer for human SCF gene obtained from GenBank AF400437 was sense primer: 5'-CAC TAA ATT GGT GGC AAA TCT TCC-3'; antisense primer: 5'-TGT GAC ACT GAC TCT GGA ATC TTT-3', and for human β-actin was synthesized as follows – sense primer: 5'-CTT CTA CAA TGA GCT GCG TG-3'; antisense primer: 5'-TCA TGA GGT AGT CAG TCA GG-3'. The PCR was performed in a thermal cycler with 27 cycles (30 s denaturation at 94°C, 1 min annealing at 60°C and 2 min extension at 72°C). Final extension was at 72°C for 7 min. By amplification SCF and β-actin in the same PCR tube we obtained data about SCF expression with reference to β-actin expression. The products were subjected to electrophoresis in 2% agarose gel and SCF mRNA expression analysed by densitometry. A representative picture of a agarose gel with β-actin and SCF is shown in Fig. 1.

SCF ELISA

The SCF protein in supernatants was measured by enzyme-linked immunoabsorbant assay (ELISA) using commercially available kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer protocol. The limit of detection for SCF protein was 9 pg/ml. All results were calculated per 1 µg of dsDNA in cells, measured by the method described by Labarca and Paigen (25).

Morphometry

A fragment of polyp tissue from each patients was analysed. Each tissue specimen was fixed in 10% buffered formalin. After fixation tissue specimens were embedded in paraffin, sections cut precisely at 4 µm and stained by haematoxylin and eosin as well as by

Table 1. Characteristics of patients with rhinosinusitis and nasal polyps

Patients with rhinosinusitis	29
Sex (men/women)	16/13
Age (years)	
Mean	42
Minimum–maximum	23–58
Patients with bronchial asthma	12
Patients with atopy	11
Patients with aspirin hypersensitivity	9
Number of polypectomies	
Median	2
Minimum–maximum	1–4

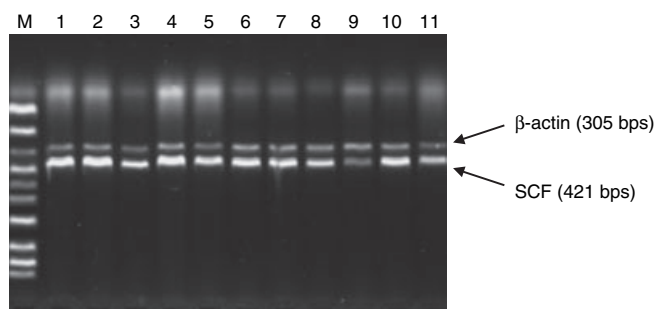


Figure 1. Stem cell factor (SCF) expression in nasal polyp epithelial cells from 11 patients assessed by reverse transcriptase polymerase chain reaction (RT-PCR). Amplified cDNA size for SCF and β -actin are shown as numbers of base pairs on the right; M, molecular size markers.

chromotrope R-2 to detecting eosinophils. Mast cells were detected in tissue specimens after staining with toluidine blue. Thickness of each section was controlled according to the method described by Weibel (26). Histological morphometry was performed by means of image analysis system consisting of a IBM – compatible computer equipped with an optical mouse, Indeo Fast card (frame grabber, true-colour, real-time), produced by Indeo (Taipei, Taiwan), and colour TV camera Panasonic (Kadoma, Japan) linked to a Carl Zeiss Jenaval microscope (Carl Zeiss Jenaval, Jena, Germany). This system was programmed (program MULTISCAN 8.08, produced by Computer Scanning Systems, Lodz, Poland) to calculate the number of objects. These objects (eosinophils and mast cells) were automatically counted and follow out with manual correction, as needed. The coloured microscopic images were saved serially in the memory of a computer, and then quantitative examinations had been carried out in two zones of a tissue. The zone above the basal membrane and that from the lower border of basal membrane to 0.18 mm depth was defined as epithelial layer (EL) and the rest of the tissue area as SL. For each tissue layer, the number of stained cells was determined in a sequence of 7–10 consecutive computer images of high power fields -0.0049 mm^2 each. The only adjustments of field were made to avoid large vessels and areas of damaged epithelium. The results were expressed as a mean number of stained cells per mm^2 .

Statistical analysis

For comparisons of SCF/ β -actin mRNA ratio and SCF production at protein level of NPEC between subgroup of patients the non-parametric Mann-Whitney *U*-statistic was used preceded by evaluation of normality. Correlation coefficients were calculated using Spearman's rank analysis method. A *P*-value lower than 0.05 was considered as statistically significant.

Results

SCF expression and clinical phenotypes

Nasal polyp epithelial cells from all patients tested ($n = 29$) demonstrated the presence of mRNA transcripts encoding SCF with the median SCF/ β -actin ratio: 0.5, range 0.1–1.5. The SCF protein was released into the supernatants of NPEC from all patients and the median

concentration was $0.3 \text{ pg}/\mu\text{g}$ DNA range 0.02–1.9. Significant correlation was noted between SCF mRNA and protein level ($r = 0.64$; $P < 0.001$).

In the whole group of patients ($n = 29$) the number of polypectomies correlated with SCF mRNA expression ($r = 0.62$; $P < 0.005$) and with the SCF protein level in

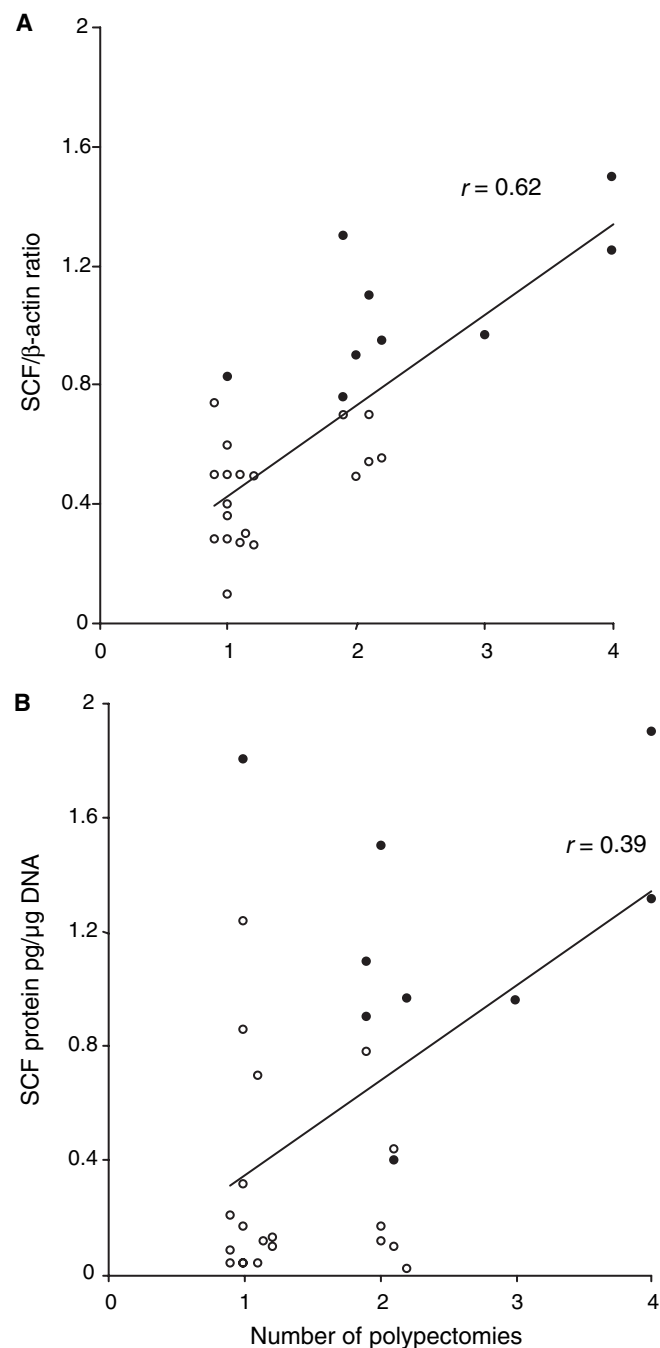


Figure 2. Correlations between number of polypectomies and (A) stem cell factor (SCF) mRNA in cultured nasal polyp epithelial cells (NPEC) ($r = 0.62$; $P < 0.005$); (B) SCF protein in supernatants of cultured NPEC ($r = 0.39$; $P < 0.05$) in the whole group of patients ($n = 29$). Open circles – aspirin-hypersensitive asthmatics; closed circles – aspirin-tolerant patients.

cell supernatants ($r = 0.39$; $P < 0.05$) (Fig. 2A,B). When correlations within different patients subpopulations were calculated the number of polypectomies was associated with SCF mRNA level ($r = 0.8$; $P < 0.05$), but not with SCF protein level ($P = 0.49$) only in the subset of AH patients with asthma ($n = 9$). No such correlations were observed in the subsets of ASA-tolerant (AT) patients ($n = 20$; $P = 0.11$), asthmatics patients with and without ASA hypersensitivity ($n = 12$; $P = 0.06$) or in nonasthmatic patients ($n = 17$; $P = 0.24$).

Nasal polyp epithelial cells obtained from AH patients ($n = 9$) showed significantly higher expression of SCF mRNA (median SCF/ β -actin ratio: 0.97, range: 0.8–1.5) than NPEC from AT patients (median SCF/ β -actin ratio: 0.5, range: 0.1–0.7; $P < 0.0001$). There was also a significant difference in SCF protein production between AH (median 1.10 pg/ μ g DNA, range: 0.4–1.9) and AT patients (median 0.1 pg/ μ g DNA, range: 0.02–1.2; $P < 0.001$) as shown in Fig. 3.

The NPECs from asthmatic patients (both AT and AH) expressed higher SCF mRNA (median SCF/ β -actin ratio: 0.9, range: 0.3–1.5 vs 0.5, range: 0.1–0.7; $P < 0.01$) and higher SCF protein concentration in supernatants (median 0.97 pg/ μ g DNA, range: 0.1–1.9 vs 0.1 pg/ μ g DNA, range: 0.02–1.24; $P < 0.001$). However, individual values of SCF mRNA and SCF protein in three asthmatics who were not ASA-sensitive were below the lowest SCF values in ASA-sensitive asthmatics group (Fig. 3). The SCF mRNA expression and SCF protein concentration were similar in patients with or without atopy (data not shown).

Cell density in nasal polyp tissue and clinical phenotypes

For the whole group of patients the number of polypectomies correlated with the density of mast cells in the SL of nasal polyp tissue ($r = 0.37$; $P < 0.05$) but not with the density of mast cells in the EL. There was no

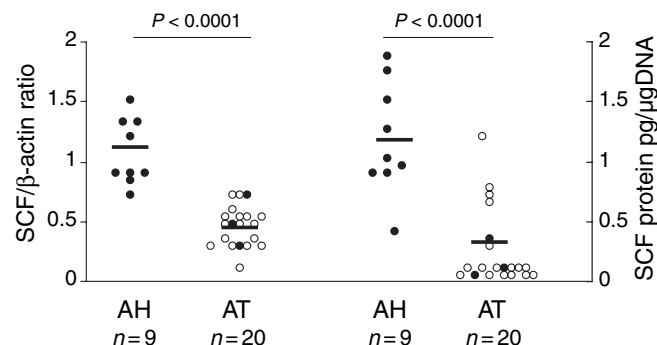


Figure 3. Stem cell factor (SCF) mRNA (SCF/ β -actin ratios) in nasal polyps epithelial cells and SCF protein production by cultured nasal polyp epithelial cells (NPEC) in aspirin-hypersensitive (AH) asthmatics and aspirin-tolerant (AT) patients. Horizontal bars represent the median values for each group. Open circles – nonasthmatic patients; closed circles – patients with bronchial asthma.

correlation between the number of polypectomies and the density of eosinophils in nasal polyps tissue.

In ASA-sensitive patients, the number of polypectomies correlated with the density of mast cells in EL ($r = 0.8$; $P < 0.01$) and SL ($r = 0.8$; $P < 0.005$) (Fig. 4A,B) and with the density of eosinophils in EL ($r = 0.7$; $P < 0.05$). Similar correlation between the number of polypectomies and the number of mast cells was found in asthmatic patients in EL ($r = 0.81$; $P < 0.001$) but not in the SL ($r = 0.57$; $P = 0.05$).

The density of mast cells was significantly higher in AH patients than in AT patients in both EL ($P < 0.005$) and SL ($P < 0.05$) of polyps. The density of eosinophils in superficial but not in SL was also increased in AH patients in comparison with AT patients (Table 2). The density of mast cells and eosinophils was significantly

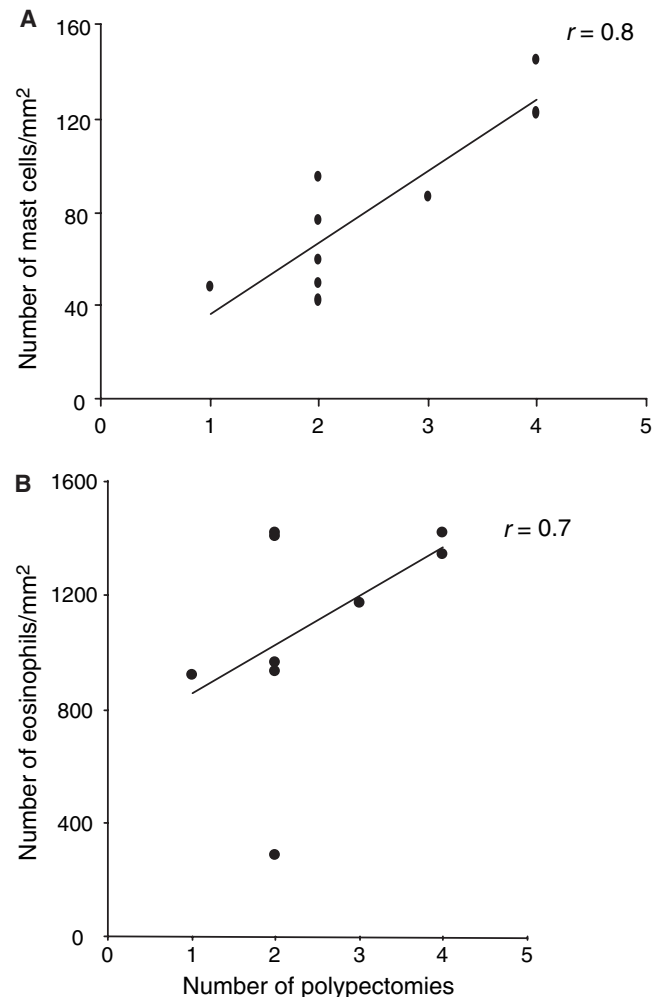


Figure 4. Correlations between the number of polypectomies and (A) the density of mast cells in epithelial and subepithelial layer (ESL) ($r = 0.8$; $P < 0.01$); (B) the density of eosinophils in ESL ($r = 0.7$; $P < 0.05$) in a subset of aspirin-sensitive asthmatics.

Table 2. Density and distribution of mast cells and eosinophils in nasal polyps cultured for epithelial cells from patients with various clinical phenotypes

Patients	Number of patients (<i>n</i>)	Mast cells (cell/mm ²)		Eosinophils (cells/mm ²)	
		ESL	SL	ESL	SL
Aspirin-sensitive	9	76.1 (41.6–145.2)**	22.5 (11.9–76.9)	1178.8 (293.2–1425.8)**	816.2 (39.8–1211.7)
Aspirin-tolerant	20	19.1 (6.5–92.1)	14.25 (4.6–40.2)	807.7 (115.4–1584.2)**	247.8 (45.3–1122.2)
AS vs AT		<i>P</i> < 0.005	<i>P</i> < 0.05	<i>P</i> = 0.05	NS
With asthma (asthma)	12	80.8 (41.6–145.2)**	24.3 (11.9–76.9)	1195.1 (293.2–1425.8)**	801.7 (39.8–1211.7)
Without asthma (WA)	17	16.2 (6.5–92.1)	12.1 (4.6–40.2)	769.2 (115.4–1584.2)	244.5 (45.3–1122.2)
Asthma vs WA		<i>P</i> < 0.005	<i>P</i> < 0.05	NS	NS
Atopic (A)	11	85.6 (33.2–145.2)*	26.2 (11.6–76.9)	1211.5 (293.2–1584.2)*	817.2 (39.8–1211.7)
Nonatopic (NA)	18	16.95 (6.5–92.1)	14.1 (4.6–40.2)	807.7 (115.4–1488.3)	215.6 (45.3–965.1)
A vs NA		<i>P</i> < 0.005	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05

**P* < 0.05, significantly different when compared with respective cells in stromal layer.

***P* < 0.01, significantly different when compared with respective cells in stromal layer.

NS, not significant; ESL, epithelial and subepithelial layer; SL, stromal layer.

Data are expressed as median (range).

higher in atopic patients than in nonatopic patients in both EL (*P* < 0.05) and SL (*P* < 0.05).

The density of mast cells was significantly higher in patients with bronchial asthma than in nonasthmatic patients in both EL (*P* < 0.005) and SL (*P* < 0.05) although there was no differences in the density of eosinophils between patients with bronchial asthma and nonasthmatic subjects.

Correlation between SCF production by cultured NPEC and inflammatory cells in nasal polyps

There was a significant correlation between SCF mRNA expression in cultured NPEC and the density of mast cells in epithelial and subepithelial layer (ESL; *r* = 0.37; *P* < 0.05) and in the SL of nasal polyps (*r* = 0.48; *P* < 0.01) (Fig. 5A,B).

Mast cell density in EL of nasal polyp tissue correlated with SCF protein level in epithelial cells supernatant (*r* = 0.51; *P* < 0.005). In AH patients, there was a significant correlation between SCF mRNA expression in cultured NPEC and the density of mast cells in SL (*r* = 0.8; *P* < 0.001). No correlations between the number of eosinophils and SCF mRNA expression or SCF protein in NPEC supernatants were found.

The number of mast cells correlated with the number of eosinophils both in the EL (*r* = 0.56; *P* < 0.001) and in the SL of nasal polyps (*r* = 0.04; *P* < 0.01). Similar correlation was found in nasal polyps from ASA-sensitive patients (*r* = 0.8; *P* < 0.01 and *r* = 0.7; *P* < 0.03 for EL and SL, respectively) and in asthmatic patients (*r* = 0.6; *P* < 0.04 and *r* = 0.69; *P* < 0.01), but not in nasal polyps from AT or nonasthmatic patients.

Discussion

In this study, the expression of SCF mRNA and SCF protein levels were assessed in cultured NPECs and

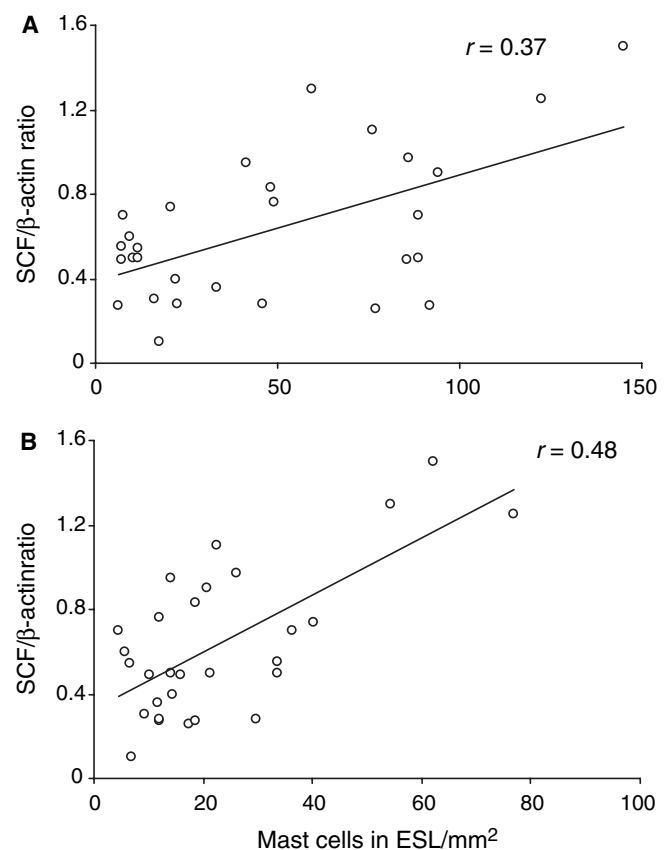


Figure 5. Correlations between stem cell factor (SCF) mRNA expression in cultured nasal polyp epithelial cells (NPEC) and the density of mast cells in epithelial and subepithelial layer (ESL) (*r* = 0.37; *P* < 0.05) (A) and in stromal layer (SL) (*r* = 0.48; *P* < 0.01) (B) of nasal polyps.

referred to clinical characteristics of patients and to inflammatory cells infiltrate in the polyp tissue. The SCF expression in epithelial cells correlated strongly with the number of polypectomies confirming a potential signifi-

cance of SCF for the pathogenesis of nasal polyposis. The SCF, the ligand for the receptor encoded by c-Kit is a major growth and differentiation factor for mast cells with strong survival promoting activity (27, 28). The SCF has also a potent chemotactic activity towards human mast cells, which altogether makes it a candidate for major molecule responsible for development of mast cells infiltration (13). Previous study demonstrated a significant increase in expression of the SCF in the nasal polyps epithelium when compared with normal nonpolyposus nasal mucosa (15). Taking together it may suggest that SCF released by epithelial cells contributes to development of tissue inflammation underlying pathogenesis of nasal polyposis.

Interestingly, the expression of SCF was strongly related to the presence of ASA-sensitive asthma. Moreover, expression of SCF both at the mRNA and protein level were significantly higher in AH asthmatics suggesting existence of the pathogenetic link with either ASA sensitivity or with bronchial asthma.

The presence of ASA sensitivity is known to be associated with particularly severe and recurrent form of nasal polyposis (29). Using CT it has been documented that rhinosinusitis in AH patients with nasal polyposis is characterized by higher thickness of hyperthrophic mucosa involving all sinuses and nasal passages (17, 29, 30). A decreased expression of cyclo-oxygenase-2 mRNA in nasal polyps tissue and PGE₂ deficiency in NPECs from AH patients were also demonstrated (31–33). It was therefore postulated that arachidonic acid metabolism abnormalities may contribute to the pathophysiology of chronic inflammation in AH patients (34). Along these lines Sousa et al. (35) documented an increased expression of LT1 receptors in nasal polyp tissue.

Respiratory form of ASA hypersensitivity is usually associated with bronchial asthma, and only few patients may have isolated upper airway disease (36). Accordingly, all ASA-sensitive patients in our study suffered from bronchial asthma, not allowing to exclude the possibility that bronchial asthma and not ASA sensitivity was the phenotype associated with the number polypectomies and with increased SCF expression. However, when the subset analysis was performed the number of polypectomies correlated with SCF expression only in a subgroup of ASA-sensitive asthmatics, but not in the whole group of

asthmatic patients (both AH and AT). Although the mean SCF level was significantly higher in asthmatic group when compared with nonasthmatics, in three asthmatic patients without ASA hypersensitivity SCF mRNA expression and SCF protein levels were below the lowest values found in ASA-sensitive patients with asthma. This data taking together suggest that ASA hypersensitivity rather than bronchial asthma was the major phenotype associated with increased SCF expression.

This is the first study referring the SCF expression in epithelial cells in culture to the histopathology of individual polyps. The expression of SCF correlated closely with the density of mast cells but not eosinophils in nasal polyp tissue indicating that SCF released from epithelial cells may contribute to the mast cells recruitment into nasal polyps and/or to their survival in the polyp tissue. The density of mast cells correlated also with the number of polypectomies implicating an important role of mast cells in the pathogenesis of nasal polyposis.

The mechanism responsible for an increased expression of SCF in NPECs from ASA-sensitive patients is not known. As the presence or absence of atopy, defined by SPT positivity to inhalant allergens was not related to SCF expression a significant role of allergic sensitization in modulation of its production seems to be unlikely. Viral infections have been associated with the onset of nasal polyposis and ASA sensitivity, thus one cannot exclude that ongoing viral infection may be responsible for increased activation of epithelial cells and release of SCF in this subset of patients (36, 37, 38).

In conclusion, we have demonstrated that severe form of nasal polyposis as observed in AH patients with asthma is associated with increased expression in cultured epithelial cells of SCF an important mast cell growth factor. Furthermore, expression of SCF in epithelial cells and the density of mast cells in polyps tissue correlated with the number of polypectomies, suggesting a pathophysiological link between SCF, mast cells and severity of nasal polyposis.

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