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Basic fibroblast growth factor in nasal polyps immunohistochemical and quantitative findings

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SUMMARY *Basic fibroblast growth factor (bFGF) is a mediator with potent mitogenic properties. Increased amounts of this mediator have been demonstrated in damaged lung tissue, and it has been suggested to increase the healing of gastro-duodenal ulcers. In order to quantify the amounts and document the localization of bFGF in nasal polyps, polyp tissue from 12 patients undergoing polypectomy was analyzed by ELISA and immunohistochemistry. Mucosa from the inferior turbinate was analyzed in the same manner for comparison. The amount of bFGF detected in polyp tissue was significantly higher than that in turbinate mucosa. The amount of bFGF was also significantly higher in the group of patients with high degree of inflammation. The immunohistochemical findings demonstrated abundant bFGF activity mainly in the glandular acini, in the epithelium, in infiltrating inflammatory cells and in the vessel walls. We propose that bFGF may contribute in a significant way to the formation of nasal polyps.*

Key words: basic fibroblast growth factor, cytokines, ELISA, immunohistochemistry, nasal polyps

INTRODUCTION

Nasal polyps are masses of edematous tissue in the nasal and paranasal cavities, usually most prominent in the middle meatus. This pathological condition is often associated with chronic respiratory disorders such as asthma, primary cilia dyskinesia, chronic sinusitis and cystic fibrosis. The impaired nasal breathing caused by the polyps may in theses cases significantly increase the suffering of the patient. It is generally recognized that nasal polyps represent a response to a persistent inflammation in the nasal mucosa. A massive infiltration of eosinophils in the polyp tissue is typical, which is why much of the current research focuses on the cell-to-cell interaction between eosinophils and the surrounding stroma. As in other inflammatory processes, the combined effects of a great number of cytokines may contribute to inflammatory cell chemotaxis in nasal polyps. E.g., interleukin-5 (IL-5) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been suggested as factors contributing to the local tissue eosinophilia (Jordana et al., 1997), while interleukin-1 (IL-1) (Liu et al., 1993) and growth factors such as transgenic growth factor α1 (TGF α1) and transgenic growth factor β1 (TGF-β1) (Elovic et al., 1994) may be of importance for the growth and maintenance of pathological tissue in the process of polyp formation.

Compared to normal mucosa, the mucosa surrounding nasal polyps reveals more frequent epithelial damage (Wladislavosky-Waserman et al., 1984), which may be indicative of a more or less continuous inflammatory stress. The accumulated eosinophils may to a significant degree contribute to the injury through the release of potent degrading enzymes, such as eosinophilic cationic protein (ECP) and major basic protein (MBP). Thus, the polyp epithelium is an environment where tissue degradation and remodelling is an ongoing process, which may trigger mechanisms involved in healing.

Basic fibroblast growth factor (bFGF), also referred to as fibroblast growth factor 2 (FGF-2), is a cytokine that stimulates the proliferation of cells of mesodermal and neuroectodermal origin, including fibroblasts and endothelial cells (Hughes and Hall, 1993). bFGF has been isolated from a variety of tissues, including neural tissue, placenta (Burgess and Maciag, 1989) and may be of great importance for hematopoesis, angiogenesis, wound healing and functioning of the nervous system (Slavin,

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1995; Bikfalvi et al., 1997). E.g., bFGF stimulates proliferation of the gastro-intestinal epithelium, which has been shown to accelerate the healing of experimental duodenal ulcers in rats (Szabo et al., 1994). Powers and co-workers demonstrated that bFGF exists in blood vessels and along the basement membrane of nasal polyps (Powers et al., 1998), and the same authors suggested that this cytokine contributes to local endothelial and epithelial cell proliferation.

The aim of the present study was to quantify and compare the amount of bFGF in nasal polyps and in non-polypous nasal mucosa from the same patients. The aim was also to document the location of this cytokine in the tissue by immunohistochemistry.

MATERIAL AND METHODS

Nasal polyps and biopsies of the ipsilateral inferior turbinate were collected from 17 patients of 33-75 (mean 51) years of age undergoing elective polypectomy at Jyväskylä Hospital. Eleven of the patients had undergone one or more previous polypectomies. Six patients had a history of asthma, and out of these four were NSAID intolerant. All patients were taking intranasal corticosteroid spray or inhalant at the time of the operation. All but three patients had a history of allergic rhinitis. From all patients, some of the excised polyps were taken for histopathological diagnosis. These samples were fixed in formalin and transferred to the Department of Pathology of the hospital, where they where embedded in paraffin, sectioned and mounted on glass slides. An independent pathologist at the hospital reviewed the slides in a light microscope and recorded the diagnosis. In addition, the degree of inflammation in the tissue was determined by semiquantification of the number of infiltrating inflammatory cells, and expressed according to an arbitrary scale, where 0 = no inflammation, 1 = slight and 2 = heavy inflammation.

Tissue specimens harvested for the analysis of bFGF were immediately packed in airtight aluminium foil and stored at -80°C until the day of analysis. From each sample, part of the tissue was taken for quantification of bFGF by ELISA, and analysis of total protein content. Remaining tissue was fixed in Tris-buffered formalin, without any enzymatic or antigen-demasking pre-processing, and embedded in paraffin for later sectioning and immunohistochemical analysis of bFGF receptor-one (bFGF R1).

Tissue specimens selected for quantification of bFGF and total protein content were weighed, thawed and transferred into glass tubes. Phosphate buffered saline, pH 7.4, was added to the tubes, ten times the sample weight for polyps; one hundred times the sample weight for nasal mucosa. The tissue and fluid mixture was homogenized for 20 seconds in a CAT X120 homogenizer (Ingenieurbüro CAT, M. Zipperer GmbH, Staufen, Germany). The homogenized material was transferred to Eppendorf tubes and centrifuged for 7 minutes at 15,000 rpm at a temperature of +4°C. Aliquots of the supernatant were taken for quantification of bFGF by ELISA and analysis of the total protein content, respectively.

ELISA

For the determination of the bFGF contents of the samples a commercially available enzyme-linked immuno-solvent assay (ELISA) kit was used (R&D System, Minneapolis, USA). The principles of this method have been described by Stanley and co-workers (Stanley et al., 1985). The procedure was carried out according to the instruction manual provided by the manufacturer. In short, 100 μl of assay dilutent and 150 μl of standard or sample was added to each well. The examination kit was covered with a sheet and incubated for 2 hours at room temperature on a horizontal microtiter plate shaker set at 500 rpm. The samples were thereafter washed four times with buffered distilled water. Then 200 μl of bFGF conjugate was added to each well, followed by incubation for two hours under a cover sheet. Again, the wells were washed four times, and 50 μl of substrate solution was added to each well, followed by one hour of incubation at room temperature. Thereafter 50 μl of amplifier solution was added to each well followed by incubation for 30 minutes at room temperature. Finally, 50 μl 2N sulphuric acid was added to each well to stop the reaction. The optical density of the wells was determined within 30 minutes using a spectrophotometer set at 490 nm and 650 nm. The content of bFGF in the samples was calculated from the standard solution in the kit, and expressed in pg/ml.

The samples for the determination of protein content underwent the same homogenization process as above, and 50 μl of the fluid phase was used.

Total protein content

The protein in the samples was solved in an alkaline medium and analyzed according to Lowry (Lowry et al., 1951). The protein content was expressed as g/liter. We were thus able to compare the amount of bFGF in polyp tissue with that in turbinate mucosa when expressed as pg bFGF /mg protein.

Immunohistochemistry

Analysis of bFGF receptor-one (bFGF R1) was done with immunoperoxidase staining. Two antibodies (AB) were used: AB1: Rabbit anti bFGF R1 (Sigma F5421), and AB2: Goat anti-rabbit biotinylated AB (Dako E4032). Tissues were sectioned and mounted on super-frost glass slides. Sections used for analyses were 4μm thick. The slides were first submersed in xylene for 2 x 2 minutes, then in OCH baths for 2 x 2 minutes in 100%, 95%, 70% and 30% solutions, respectively, followed by a 10 minutes rinse in water. Thereafter the specimen were soaked in 3% peroxidase in methanol 1:4 for 30 minutes, followed by a 20 minutes incubation with goat serum 1% in phosphate buffered saline (pH 7.4). Rabbit anti-bFGF R1 was then added and the specimens were kept under cover for 2 hours. This was followed by three five-minute rinses in phosphate buffered saline after which the second antibody was added (goat anti-rabbit biotinylated) and incubated on the slides for 1 hour.

The avidin biotinylated horseradish peroxidase macromolecular complex (ABC) was used according to Hsu et al. (Hsu et al., 1981). Again, the slides were washed three times five minutes in phosphate buffered saline after which the ABC solution (Vec-

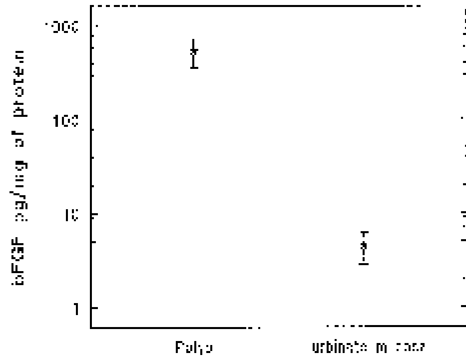

Figure 1. The concentration of bFGF in relation to the total protein content (pg bFGF/mg protein) in polyps and turbinate mucosa, respectively. The concentration of bFGF was significantly higher in polyp tissue ($p < 0.001$, Wilcoxons signed ranks test).

tastain® ABC kit, Vector labs, Burlingame, CA, USA) was added and kept on the slides for 30 minutes, followed by another 3 x 5 minutes rinse in phosphate buffered saline. The slides were then soaked into a 3-amino-9-ethyl carbazole (AEC) solution with peroxide for 7 minutes and a brown color developed. Finally, the slides were rinsed in tap water for 2 minutes. Counterstaining was carried out with Mayers solution for 25 seconds after which the slides were rinsed under running water for 10 minutes. Blue pigmentation of nuclei could then be confirmed by examination in a light microscope. The slides were then immediately mounted. In order to rule out false positive results, additional slides from every second specimen underwent a similar process without the adding of the active staining substance. From each tissue specimen slides were also taken for hematoxyline-eosin staining and identification of eosinophilic granulocytes. The day after the staining procedure, all slides were examined and photographed in a light microscope.

Statistics

Differences regarding amount of bFGF R1 in polyps and turbinate mucosa in all patients were analyzed by the Wilcoxon signed-rank test. Differences between groups of patients regarding degree of inflammation, occurrence of asthma and previous polypectomies were analyzed using the Mann-Whitney U-test.

Figure 3. The concentration of bFGF in polyp tissue from patients with asthma (A(+)) and without asthma (A(-)). The difference is not significant.

RESULTS

Histopathology

A diagnosis of eosinophilic nasal polyposis could be confirmed for all patients. The degree of inflammation in the polyp tissue was defined as “2” (heavy) for eleven patients, and as “1” (slight) for the remaining six.

Elisa

In general, the amounts of bFGF receptor protein detected were higher than expected, ranging from 186 to 918 pg/ml, thus exceeding the upper range of the standard curve. The amount of bFGF detected in polyp tissue was significantly higher than that in turbinate mucosa (Figure 1). Further, the concentration of bFGF in polyp tissue from patients with heavy inflammation (grade 2) was significantly higher in than in polyps from patients with slight inflammation (grade 1). However, this was not true for nasal turbinate mucosa (Figure 2). There was a trend

Figure 2. The concentration of bFGF in polyp tissue and nasal turbinate mucosa with slight (grade 1) vs. heavy (grade 2) inflammation. N(-): nasal mucosa with slight inflammation, N(+):nasal mucosa with heavy inflammation, P(-): polyp tissue with slight inflammation, P(+):polyp tissue with heavy inflammation. The difference between the two groups of inflammation is significant for polyp tissue ($p = 0.007$, Mann-Whitney U-test), but not for nasal turbinate mucosa.

Figure 4. The concentration of bFGF in polyp tissue from patients who had undergone one or more previous polypectomies and in patients who had not undergone such a procedure. O(-): no previous polypectomy, O (+): one or more previous polypectomies. The difference is not significant.

towards higher bFGF in polyps from patients who had asthma (Figure 3), or who had undergone previous polypectomies (Figure 4), but these differences did not meet significance.

Immunohistochemistry

The immunohistochemical experiment demonstrated abundant bFGF activity in both polyp and turbinate tissue. bFGF was expressed primarily in the glandular acini, the epithelium, in infiltrating inflammatory cells and in the vessel walls (Figures 5 and 6). Most of the staining activity was localized to the basal membrane. There was no marked difference between polyps and turbinate

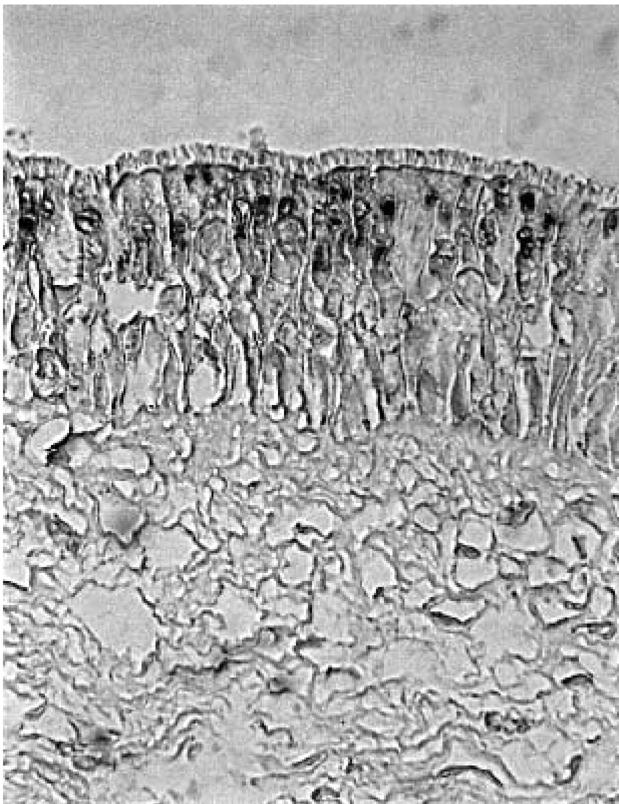

Figure 5. Nasal polyp tissue stained for bFGF R1 activity. The staining is confined to localized parts of the epithelium. 400 x original magnification.

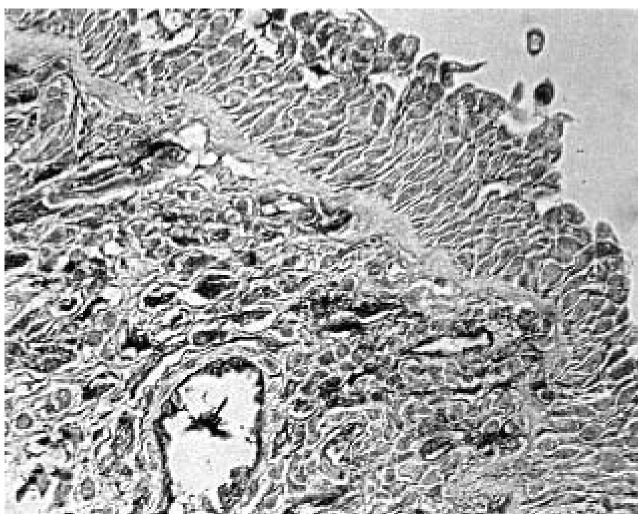

Figure 6. Nasal polyp with abundant bFGF R1 activity around blood vessels and glands. 400 x original magnification.

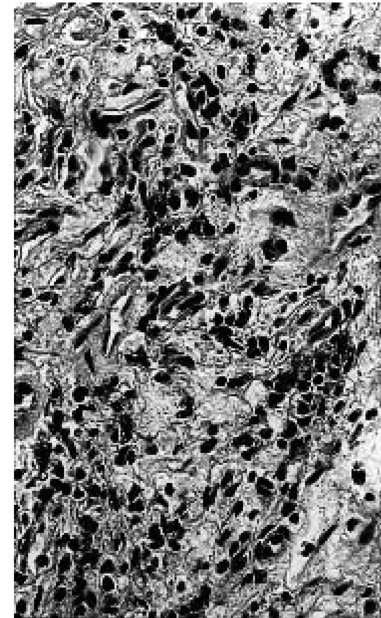

Figure 7. Nasal polyp tissue stained with hematoxylin-eosin. Numerous infiltrating eosinophilic granulocytes can be seen, mainly around blood vessels and glands. 400 x original magnification.

mucosa apart from the fact that there was only scarce staining for bFGF in the polyp edema. No staining was seen in control slides. Slides stained with hematoxylin-eosin demonstrated heavy infiltration of eosinophils in all polyps, mainly around vessels and glands (Figure 7).

DISCUSSION

We could detect bFGF activity in the glandular acini, in the epithelium and in the infiltrating inflammatory cells. This is in line with the findings of Powers and co-workers who demonstrated bFGF mRNA in blood vessels, epithelial cells and infiltrating mononuclear cells, using Northern blot analysis (Powers et al., 1998). Our findings also agree with other studies on bFGF in respiratory epithelium. E.g., in developing rat fetal lung bFGF is localized to cells of the airway epithelium, basement membrane and extracellular matrix (Sannes et al., 1992). In tissue sections from a patient who died following acute lung injury, bFGF was found in macrophages in areas of fibroblastic and vascular proliferation (Henke et al., 1991). Whether the infiltrating cells positive for bFGF in our study consisted of leukocytes, macrophages, mast cells or other inflammation markers could however not be determined.

The amount of bFGF was significantly higher in polyp tissue than in inferior turbinate mucosa. This result differs from that of Ishibashi and co-workers who found low amounts of acidic and basic FGF in both polyp tissue and nasal mucosa, using reverse transcription-polymerase chain reaction (RT-PCR) (Ishibashi et al., 1998). The nasal mucosa in their study was hyperplastic, which could explain the lack of difference between the two tissue types, but not why only low amounts of the cytokine were detected.

In our study, the amount of bFGF was higher in polyp tissue from patients with more pronounced inflammation. Since inflammation may be regarded as a key factor in nasal polyposis,

this indicates that bFGF indeed is involved in the process of polyp formation, although it is difficult to determine what is cause and effect from our findings.

The role of bFGF and other growth factors in nasal polyposis

Nasal polyposis is usually regarded as a multifactorial disease, and a large number of cytokines have been suggested to be of importance for the pathogenesis. Lee and co-workers compared the expression of interleukin-1β (IL-1β), IL-4, IL-5, IL-6, IL-8, interferon-γ (IFN-γ) and TGF-β mRNAs in polyps from 14 patients with turbinate mucosa from healthy volunteers (Lee et al., 1998). With the exception of IL-1β, all these cytokine genes were detected more frequently in polyp tissue. IL-5 has been appointed as the cytokine of highest importance for the eosinophilia that is found in most nasal polyps (Jordana et al., 1997). Several growth factors are important for the process of tissue repair which is closely linked to virtually all types of inflammation, and which may be of particular importance in polyp formation. TGF-β1 is produced by eosinophils in nasal polyps and may contribute to local thickening of the basal membrane, stromal fibrosis and epithelial hyperplasia (Elovic et al., 1994). Insulin-like growth factor 1 (IGF-1) is a trophic factor that may induce proliferation of several types of tissue cells. This factor is found in high concentrations in nasal polyps and ethmoidal mucosal cells, and has been suggested to contribute to polyp growth (Petruson et al., 1988). In addition, Coste and co-workers have proposed that epithelial cell proliferation in nasal polyps could be up-regulated by platelet-derived growth factor (PDGF). They detected intense PDGF expression in hyperplastic and metaplastic areas of nasal polyp epithelium, and suggested that PDGF in polyps could be produced by macrophages and eosinophils (Coste et al., 1996 a; Coste et al., 1996 b). It is in this highly complex of interacting mediators that we suggest that the stimulatory effects of bFGF on angiogenesis, wound healing and tissue repair also will play a role.

CONCLUSION

In this study, we demonstrated an increased amount of bFGF in nasal polyps as compared to turbinate tissue. The potent mitogenic effect of bFGF on human stromal cells, documented in previous studies on tissue damage, may well account for several components in polyp formation. Specifically, the triggering effects of the continuous inflammatory epithelial injury could lead to an increased secretion of bFGF from infiltrating macrophages. This, in combination with the effects other mediators, may contribute to accelerated reproduction of vascular and epithelial cells. The distension forces caused by the leaking tissue fluid may then lead to the bulging polyp formation.

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