Differential Expression of Extracellular Matrix Components in Nasal Polyp Endotypes

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Abstract

Background: Chronic rhinosinusitis is a difficult-to-treat disease that is often characterized by recurrent nasal polyp (NP) growth following surgical removal. The disease has been separated into distinct phenotypes based on cellular infiltrate or underlying physiological mechanisms. NPs are composed in part of an inflammatory cellular infiltrate, blood vessels, and a large amount of extracellular matrix (ECM). Despite the recognition of prominent ECM deposition, few studies have examined the components in detail and how they might differ with disease state.

Objective: The purpose of this study was to quantitate the expression of ECM components in NPs.

Methods: NPs were stained with pico-sirius red to determine total collagen content, and immunofluorescence was used to detect collagen I, collagen III, collagen IV, fibronectin, and laminin. Expression of each was quantitated and analyzed in relation to rhinosinusitis phenotype and separately as a function of polyp eosinophil number.

Results: When analyzed by phenotype, collagen I, collagen III, and fibronectin were expressed at the highest levels in noneosinophilic sinus disease. Collagen IV was not different among any groups, and its location was found predominately around vessels. When analyzed as a function of polyp eosinophil number, total collagen and collagen III showed a significant inverse correlation.

Conclusions: NP ECM composition differs with disease state with higher expression in cases where eosinophil levels are low. This suggests that in eosinophilic polyps there is a loss of matrix deposition either through break down or a failure to produce the essential components. Understanding these differences may identify new therapeutic targets.

Keywords

aspirin-exacerbated respiratory disease, chronic rhinosinusitis, extracellular matrix, eosinophils, nasal polyps, collagen, fibronectin, laminin, allergic fungal sinusitis, vessels

Introduction

Chronic rhinosinusitis (CRS) is an increasingly important medical problem with a significant adverse impact on patient's quality of life and well-being and for which current therapies often prove inadequate. Current guidelines divide CRS into 2 subsets defined by the presence or absence of nasal polyps (NPs): CRS with NP (CRSwNP) and CRS without NP (CRSSNP).¹ Factors driving this distinction are the significant association between NPs and the presence of tissue eosinophilia.^{2,3} Although this trend is true in Western cultures, not all polyps have eosinophils, and in Asian populations, non-eosinophilic or neutrophilic polyps are the dominant type.^{4,5} Ultimately, this leads to the questions regarding

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treatment and prognosis as to whether it is better to consider CRS as multiple diseases based on the inflammatory infiltrate or 2 diseases defined based upon the presence or absence of NPs. To answer these questions, better understanding of CRS phenotypes and the characteristics that distinguish them is needed.

Many studies have examined the infiltrating cell populations into NP and mediators that are secreted within the tissue.^{3,4,6} A common feature of CRS is the frequent reoccurrence of NP following surgical removal. This suggests an ongoing remodeling process that allows for the rapid growth of fibrotic cells and deposition of factors involved in extracellular matrix (ECM) formation. One study has suggested that the process of remodeling occurs in parallel with inflammation rather than subsequent to ongoing inflammation.⁷ ECM includes interstitial matrix and the basement membrane located just under the epithelial layer. Components of ECM include collagen, laminin, fibronectin, and elastin. Few studies have examined ECM deposition in NPs, and the focus has generally been on total collagen content or fibronectin expression.⁸⁻¹⁰

The current studies were performed to assess the quantity and distribution of ECM components in NPs. The components measured included collagen I, collagen III, collagen IV, fibronectin, and laminin. In particular, we investigated ECM components in relation to polyp phenotypes that have been previously identified, and we also analyzed the data of ECM deposition as a function of eosinophil number in the polyp tissue without regard to phenotype.

Methods

Subjects

NP tissue was obtained from subjects referred to the University of Virginia Health System for sinus surgery under a protocol approved by the University of Virginia Institutional Review Board. Control tissue was harvested from the sinus cavities of patients undergoing surgery that required access to their paranasal sinuses for reasons other than chronic sinusitis (eg, orbital decompression, cerebrospinal fluid leak repair, or transphenoidal pituitary surgery). Eosinophilic sinusitis (E-CRS) was histologically defined as previously described based upon the presence of ≥ 5 eosinophils/400 $\times$ high-powered field (hpf).¹¹ Noneosinophilic CRS (NE-CRS) was defined by expression of < 5 eosinophils/400 $\times$ hpf. Aspirin-exacerbated respiratory disease (AERD) was defined by a compelling history involving a hypersensitivity reaction within 1 to 3 hours of ingestion of either aspirin or another nonsteroidal anti-inflammatory drug.¹² The diagnosis of allergic fungal sinusitis required the presence of criteria as defined by Bent and Kuhn¹³ including type I

hypersensitivity to fungus, NPs, distinct computed tomography scan findings, eosinophilic ("allergic") mucin, and positive fungal stain. NPs were obtained from 33 subjects including 9 with E-CRS, 9 with AERD, 9 with NE-CRS, and 6 with AFS.

Histological Scoring

Polyp tissue was fixed in 4% paraformaldehyde, paraffin embedded, and sectioned by the Histology Core Laboratory of the University of Virginia. As previously described,^{11,14} NPs were scored for eosinophilia based upon the number of eosinophils in hematoxylin and eosin-stained sections. Sections were examined under 400 $\times$ magnification in a blinded fashion, and positive cells were counted in 4 random sections for each sample with the final number being the average number of cells per 4 hpfs.

Picro-Sirius Red Collagen Staining

Samples were deparaffinized and hydrated to distilled water. Nuclei were stained with Weigert's hematoxylin (Sigma, St Louis, MO) for 8 minutes followed by 10 minutes in distilled water to rinse the slides. Sections were placed in picro-sirius red solution (0.5 g Direct Red 80 (Sigma) in 500 mL saturated aqueous picric acid (Ricca Chemical Company, Arlington, TX) for 1 hour and then washed in 2 changes of acidified water (5 mL glacial acetic acid in 1 L distilled water) followed by dehydration and mounting. Sections were imaged with bright-field and circularly polarized microscopy at the University of Virginia microscopy core. Analysis of collagen content was performed using Image J software (National Institutes of Health, Bethesda, MD) by calculating the percent of collagen staining to the total tissue area. Three random sections for each polyp were used to determine the average collagen staining per sample.

Immunofluorescence Staining

For immunofluorescence, heat-induced antigen retrieval was performed by heating sections in a pressure cooker for 20 minutes in citrate buffer (Abcam, Cambridge, MA). Slides were washed and blocked using 1% bovine serum albumin, 10% goat serum (Sigma, St. Louis, MO), and Fc Block 1 μ g (BD Pharmingen, Sparks, MD) for 2 hours. Specific staining was performed using a 1:100 dilution of a rabbit anti-human antibody (Abcam) for 16 hours at 4°C to the following targets: laminin (ab11575), fibronectin (ab2413), collagen I (ab34710), collagen III (ab7778), and collagen IV (ab6586). Sections were rinsed and then incubated with secondary allophycocyanin goat anti-rabbit IgG (1:200, Life Technologies, Grand Island, NY) for 1 hour

at room temperature. Nuclei were stained with 100 ng/mL DAPI (4', 6-diamidino-2-phenylindole, Life Technologies) for 30 minutes at room temperature. Samples were washed in phosphate-buffered saline and aqueous mounted with VectaMount AQ (Vector Laboratories, Burlington, CA). The samples were analyzed using an EVOS FL Auto microscope (Life Technologies) and positive staining scored in a blinded fashion as described above taking the average of 4 fields per slide.

Statistical Analyses

Control and CRS cohorts were compared using non-parametric unpaired *t* tests. Correlation analysis was performed using Spearman's correlation by comparing ECM components as a function of eosinophil number. A *P* value of $<.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA).

Results

ECM Content Based on Disease Phenotype

NP tissue was stained and quantitated for ECM components: total collagen, collagen I, collagen III, collagen IV, fibronectin, and laminin. Representative images are presented in Figure 1 for each stain. As described in our previous studies,¹¹ polyps were separated based on

defined phenotypes, and the amount of each ECM component was compared between groups (Table 1). The total collagen content as measured by picro-sirius red staining was highest in control tissue ($P < .005$) when compared to the different polyp phenotypes (Table 1). Collagen I, collagen III, and fibronectin staining were higher in the NE-CRS group when compared to the E-CRS, AERD, or AFS groups ($P < .03$). The common feature of the later 3 groups is that they are all characterized by an eosinophilic infiltrate. Collagen I and collagen III had a similar tissue distribution being found surrounding vessels and in the interstitial areas. Fibronectin was mainly found distributed in the interstitial areas of the tissue. Collagen IV staining did not differ among any of the groups analyzed (Table 1). Examination of the staining in each polyp type showed intense localization of staining around vessels with some diffuse interstitial and little basement membrane staining (Figure 2). Laminin expression was very low in all polyp types so quantification was not performed; however, when it was observed, it was found along vessels and the basement membrane (Supplementary Figure 1).

Treatment of ECM as a Function of Eosinophil Number

The above observations of ECM content based on disease phenotype indicated that an inverse correlation

Figure 1. Representative images of extracellular matrix components in nasal polyps. Nasal polyps from subjects with different phenotypes of chronic rhinosinusitis were examined for extracellular matrix content by direct staining or through immunofluorescence. For immunofluorescence, a primary antibody directed against the various matrix components and an APC-labeled secondary antibody (red) with DAPI (4', 6-diamidino-2-phenylindole) nuclear stain (blue) was used. Representative examples of each matrix component tested are shown. A, Sirius red. B, Isotype control. C, Collagen I. D, Collagen III. E, Collagen IV. F, Fibronectin (For interpretation of the references to colours in this figure legend, refer to the online version of this article).

Table 1. ECM Content as a Function of Disease Phenotype.

	Control (n = 6)	NE-CRS (n = 9)	E-CRS (n = 9)	AERD (n = 9)	AFS (n = 6)
Total collagen	38.4 ± 5.5	12.1 ± 2.7*	11.0 ± 1.3*	6.8 ± 0.7*	7.8 ± 2.2*
Col I	7.5 ± 1.2	11.9 ± 1.7	5.9 ± 1.3**	6.1 ± 1.1**	4.5 ± 1.2**
Col III	19.6 ± 2.2	16.7 ± 1.7	7.6 ± 1.2*	11.3 ± 1.6*	8.6 ± 0.8*
Col IV	5.9 ± 0.9	7.3 ± 0.9	3.7 ± 0.9	5.8 ± 0.8	4.8 ± 0.7
Fibronectin	10.9 ± 0.9	12.1 ± 1.3	7.9 ± 1.6	7.2 ± 1.0***	6.2 ± 0.7*

Abbreviations: AERD, aspirin-exacerbated respiratory disease; AFS, allergic fungal sinusitis; Col, collagen; E-CRS, eosinophilic chronic rhinosinusitis; NE-CRS, noneosinophilic chronic rhinosinusitis.

* $P < .005$ in comparison to control tissue.

** $P < .02$ in comparison to NE-CRS.

*** $P < .03$ in comparison to control.

Figure 2. Collagen IV staining of vessels in nasal polyps. For immunofluorescence, a primary antibody directed against collagen IV and an APC-labeled secondary antibody (red) with DAPI nuclear stain (blue) was used. White arrows indicate staining around vessels. A, Control tissue. B, NE-CRS. C, E-CRS. D, AERD (For interpretation of the references to colours in this figure legend, refer to the online version of this article).

might exist between eosinophil number and ECM content. To test this, the average number of eosinophils per hpf was determined for each polyp and correlated with the individual ECM components. Total collagen ($r = -.70$, $P < .001$) and collagen III ($r = -.38$, $P < .02$) both showed a significant inverse relationship in terms of quantity and eosinophil number (Figure 3). A similar inverse relationship was observed for collagen I and fibronectin, but these did not reach statistical significance.

Discussion

The current studies were undertaken to determine the levels and distribution patterns of ECM components in NPs. In addition, we were interested in how they correlated with disease state. Using picro-sirius red staining for total collagen, we identified the highest levels in control tissue followed by NE-CRS (Table 1). The results of this study are in accord with the findings of Meng et al. who also found the highest levels of collagen staining in

Figure 3. Correlation of collagen content and eosinophil number. Eosinophil numbers were counted for each polyp and compared to collagen content as measured by area fraction for total collagen and mean gray volume for individual components with correlations determined using Spearman's rank sum. A, Total collagen by eosinophil number. B, Collagen III by eosinophil number.

control tissue when compared to CRSwNP.⁷ The higher collagen content in the control tissue is not surprising as it is composed of basement membrane and epithelium, while polyps are edematous which would dilute the collagen content. That there are differences in collagen content in the polyps of the different phenotypes is the more important finding.

When specific collagen components were examined in the disease cohorts, collagen I and collagen III expression was found to be the highest in the NE-CRS group with no difference found in any of the other polyp groups (Table 1). Expression of these collagen components was also lower in the control group, in particular collagen I which was no different than the E-CRS, AERD, or AFS groups. Collagen I and collagen III are often found in association with each other, most commonly in connective tissues and fibrotic lesions.¹⁵ These collagens form long thin fibers that cross-link with each other to create dense networks that provide structural support.¹⁵ That they are overexpressed in NE-CRS polyps fits with descriptions of them having less edema and being fibrous bundles.^{4,5,11} Our finding of higher fibronectin in NE-CRS subjects compared to the other CRS groups is consistent with the results that were reported by Shin et al.¹⁶ who found the highest levels of fibronectin RNA and protein in noneosinophilic polyps from Korean subjects. There was no difference in collagen IV expression among any of the groups. Expression was localized to areas surrounding vessels (Figure 2) which is consistent with reports of expression in the vascular basement membrane.^{17,18}

The data on ECM content being highest in the NE-CRS group and lower in the other cohorts that contained eosinophils suggested that there might be an inverse relationship between ECM and eosinophil number. Eosinophils were counted in all samples and treated as a continuous variable rather than separated

into predefined groups which can be arbitrary. A significant negative correlation was found for total collagen and collagen III expression as a function of eosinophil number (Figure 3). A previous study correlated eosinophil numbers in the polyp with fibronectin and found a significant negative correlation between the two.¹⁹ We observed a similar result in terms of negative correlation for fibronectin though our data did not reach statistical significance (data not shown). A positive correlation between fibroblast proliferation and fibronectin immunoreactivity has also been observed⁸ that suggests NE-CRS polyps may have more fibroblast activity which is lost as eosinophils infiltrate the sinus tissue.

There are several limitations to this study. ECM is complex and composed of many components of which a limited number were examined in this study. Others may also have been altered in their expression and could be equally or more important in the disease process. RNA microarrays could be used to look at global dysregulation of ECM components or single-cell RNA sequencing to identify individual cell types where ECM expression was changed. These studies are also not able to distinguish whether the differences observed are a consequence of upstream factors driving CRS and NP formation or if it is the change in ECM expression itself that is critical to this process. This could be addressed in future studies using isolated NP fibroblasts or tissue explants and stimulating them with various cytokines to determine how this alters ECM production.

In summary, the composition of ECM in NPs differs depending on the disease state with highest expression seen in cases where tissue eosinophil numbers are low. This suggests that in eosinophilic polyps, there is a loss of matrix deposition either through enhanced break down of the ECM by proteases or a failure to produce the essential components. The result is an increase in tissue edema that occurs in more severe cases of CRS.

New therapeutic targets could be designed to prevent the remodeling process that occurs.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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