tained Cry j 1-Cry j 2 fusion protein in the immunoregulatory liposome showed suppression of IgE and IgG antibody responses after being challenged with the allergens. Furthermore, oral administration of the vaccine showed efficient suppression of IgE antibody production.[74]

CONCLUSIONS

The standardization of a vaccine enables us to compare the results from varied clinical trials with respect to dose, clinical effects, and changes in biological parameters. Many reports have shown positive clinical therapeutic effects and suppressed effector/inflammatory responses. It is considered that IL10-producing Tr1 and/or adaptive or induced Treg cells may be involved in the suppression of the antigen-specific Th2-responses and local inflammation. However, how immunotherapy induces suppressor cells like Tr1 and Treg cells remains unclear, although the involvement of mucosal dendritic cells has been proposed. High-quality clinical studies are indispensable to clarify the therapeutic biomarkers and the mechanisms of induction of suppressor cells, and the resultant data from the studies may enable us to develop safer and more effective immunotherapy through the modification of the allergens, optimum dose, or administration regimen of a vaccine.

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綜　説

スギ花粉症に対する早期介入の取り組み

岡本美孝
おかもとよしたか

患者数が増加し，かつ自然改善が少なく，特に小児で発症した患児は多くが改善のないまま成人に移行しているスギ花粉症に対しては，早期介入による対応が重要である。発症の遺伝要因としてmatrix metalloproteinase-9遺伝子の関与と，さらに1次介入，2次介入，3次介入の手段として可能性が期待される，抗原回避，抗原特異的免疫療法，薬物療法，機能性食品療法を取り上げ，これまでの検討結果を概説した。今後，具体的な介入法について多くの質の高い臨床試験が必要である。

キーワード：スギ花粉症，早期介入，免疫治療，薬物治療，機能性食品

はじめに：―スギ花粉症の現状

世界各地でアレルギー性鼻炎患者の増加が指摘されているが，わが国のアレルギー性鼻炎の特徴はなんと言ってもスギ花粉症の存在である。2008年に全国の耳鼻咽喉科医とその家族を対象に行われたアンケート調査ではスギ花粉症の国民の有症率は25.6％と，10年前に行われた同一方法での調査結果と比較して10％以上増加していることが報告された[1,2]。調査対象の選定にbiasがかかっていること，アンケートの回収率が低いといった指摘もあるが，診断に関する信頼度が高い全国調査の結果として注目されている。

一般住民を対象としたアレルギー性鼻炎の有病率の調査では，問診のみではfalse positiveの割合が高くなり，IgE抗体調査と丁寧な問診票を用いることが精度の向上に不可欠である[3]。2005年に当教室で行ったIgE抗体調査も含めた山梨県農村部の4小学校の全校生徒を対象に行った調査では，スギ花粉症感作率は57％，有病率は眼症状の合併も条件に含めた厳しい基準でも20％に達していた。一方，1995年以降千葉県房総半島の丸山地区で，40歳以上の中・高年者を対象に行っているアレルギー性鼻炎の検診結果からも，40歳代ではスギ花粉に対する感作率は45％を越えている。また，高齢になれば感作率は低下するとはいえ，スギ花粉感作陽性者を1995年から13年間追ってみると，60歳代では13年間の経過で50％が陰性化するものの，50歳代では約15％，40歳代では13年間でも陰性化する割合は5％以下であった（図1）[4]。

さらに，スギ花粉症患者の長期経過をみるために，1970～1995年に当科で診断・治療を受けたスギ花粉症患者のうち，当科での再診・再検査した患者111名の平均15年後の改善率は，抗原特異的免疫療法（減感作療法）群では，70％に達するものの薬物治療群では40％と低く（図2），特に小児では寛解例はなく，改善も軽度改善で20％のみであり，多くがそのまま成人に移行していた[5,6]。

スギ花粉症に対する早期介入

このように，感作率，有病率が増加し続け，かつ自然改善が少ないスギ花粉症に対しては，特に早期介入による対応が必要である[7]。早期介入は，アトピー素因を有する者に対して，スギ花粉特異的IgE産生を防ぐための一次介入，IgE産生が始まった者に対してスギ花粉症の発症を防ぐため二次介入，スギ花粉症の発症がみられた者に対してその重症化を防ぐ三次介入に分けられる（図3）。介入手段として，様々な開発の取り組みが行われているワクチンや抗体療法などもあるが，有効性が臨床試験で認められても，早期治療法として標準化されるには少なくとも今後10年以上の期間が必要であろう。現状での対応として，抗原回避，抗原特異的免疫療法（減感作療法），薬物療法，機能性食品が可能性を持つ

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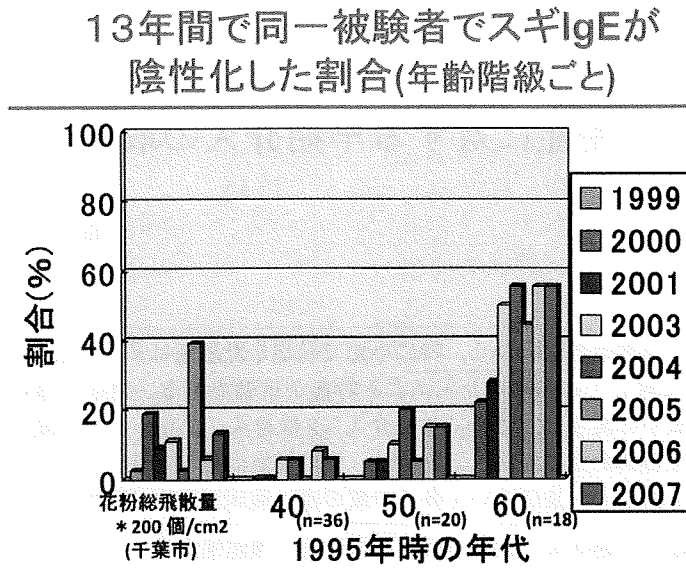


図1　1995年のスギ花粉感作陽性者の13年間での陰性化の割合（文献4から改編）。

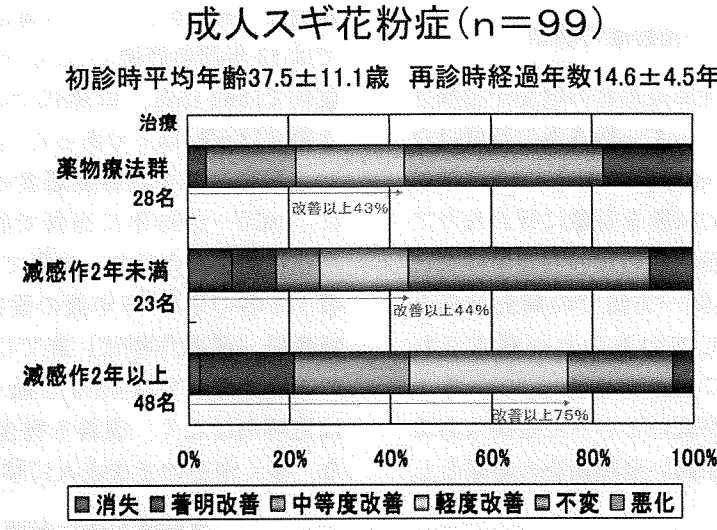
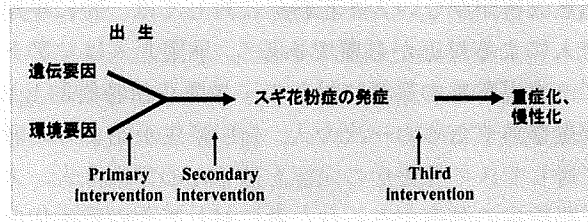


図2　成人スギ花粉症患者の長期経過（文献6から）。



・抗原回避
・薬物治療
・抗原特異的免疫治療(減感作療法)
・プロバイオティクス
・ワクチン
・その他、感染制御など

図3　スギ花粉症に対する早期介入
1次介入，2次介入，3次介入から成る。

介入手段として挙げられる。

スギ花粉症の遺伝要素

スギ花粉症の発症には，様々な遺伝要因と環境要因が関与するが，早期介入の検討では特に遺伝要因を明らかにして，介入の対象を絞って取り組みが行えれば効率も良いと考えられる。そこで，matrix metalloproteinase（MMP）-9に注目して千葉大学公衆衛生学教室，小児病態学と共同で検討を行った。MMP-9は小児アトピー型喘息患児でイントロン4（SNP-1）とエクソン12（SNP-2）の解析から感受性遺伝子としてすでに報告されている[8]が，山梨県

表1　MMP9とスギ花粉症との相関（山梨）

	患者群		対照群		χ^2	P-value
SNP1	GG	GG+GT	GG	GG+GT		
	36	4	37	14	4.302	0.038
SNP2	GG	GA+AA	GG	GA+AA		
	33	7	32	19	4.287	0.038

表2　スギIgE陽性・陰性の検討

	SNP2	
	GG	GA+AA
現在50歳代	16	14
13年連続陰性	78	26
13年間で1度でも陽性		
	0.02	
60歳代	49	24
13年連続陰性	70	29
13年間で1度でも陽性		
	0.61	
70歳代	59	31
13年連続陰性	44	17
13年間で1度でも陽性		
	0.39	

農村部の小学生270名，千葉県の小学生400名を対象に，血液，頬粘膜擦過片を用いて同様の検討を行った。

アレルギー性鼻炎の診断は，特異的IgE抗体が陽性で，明らかな症状を有するもの，対象とした正常群は，総IgE値が100IU/ml以下で，検討したダニや代表的花粉アレルゲンのCAP-RAST値がすべて陰性でかつアレルギー性鼻炎，喘息，アトピー性皮膚炎などアレルギー疾患の既往がないものとした。その結果，スギ花粉感作，スギ花粉症発症にこれらSNP-1，SNP-2のいずれもが感作性遺伝子であることが示唆された。まったく異なった地域の小学生を対象に行った調査で同様の結果が得られたことは，MMP-9遺伝子の関与をより示す結果と考えられた（表1）。ただ，ダニ通年性アレルギー性鼻炎については，ダニ感作にも発症にも有意な関連はみられなかった。喘息とアレルギー性鼻炎との発症機序，病態の違いを示すものと考えられる。

一方，前述した千葉県丸山地区の中・高年の住民を対象に2007年に同意が得られた約1,300名を対象に同様なSNP-1，SNP-2の解析を行った。ただ，成人，特に中高年者での解析にあたっては，現在は感作や症状がなくても過去には存在していた可能性があること，さらにスギ花粉抗体価は季節変動を示し，かつその年の花粉飛散数によって抗体が陽性，陰性に転化もみられること，自然改善の可能性があることから，スギ花粉感作陽性者，陰性者，花粉症患者，正常者の区別が問題となる。幸い13年間のデータを利用することが可能であるため，正常者群として13年間にわたりスギIgE抗体価が陰性であり症状がまったくないものとし，スギ花粉抗体陽性は13年間に一度でもスギIgEが陽性であったもの，スギ花粉症患者は2シーズン以上花粉飛散期に症状がみられたものとして解析を行うことで，中高年者での判定の誤差を少なくできると考えられた。その結果，スギ花粉感作，あるいは発症にいずれのSNPも小児とは異なり関連はみられなかった。しかし，対象者を年代別に分けて検討すると1995年に50歳代，60歳代であったものと異なり，40歳代であったものでは，スギ花粉感作に小児と同様にスギ花粉の感作に関連がみられた（表2）。このことから，高齢者と小児でスギ花粉に対する感作や花粉症発症の背景には違いがあること，特に中間層とも言える40歳代では関連がみられたことは，現在増加している小児から青壮年者のスギ花粉症と高齢者のスギ花粉症の発症には遺伝子の関与が異なることが示唆される。

抗原回避

近年のスギ花粉症患者の増加には，飛散スギ花粉数の増加が発症の最も重要な環境要因と考えられている。同時に，代表的Ⅰ型アレルギー疾患であるスギ花粉症では，花粉曝露の回避は治療の基本となる患者への指導として位置づけられている[2)]。

2005年のスギ・ヒノキ花粉飛散後に，毎年の花粉飛散数が異なり，かつ人の流入が比較的少ないと

表3　通年性アレルギー性鼻炎の治療とスギ花粉症発症

	通年性アレルギー性鼻炎の 治療前に花粉症発症	治療後に花粉症発症
薬物療法群	7/16 (43.8%)	5/9 (55.6%)
免疫療法群 (2年未満)	0/13 (0%)	4/13 (30.8%)
免疫療法群 (2年以上)	10/26 (38.5%)	5/16 (31.3%)

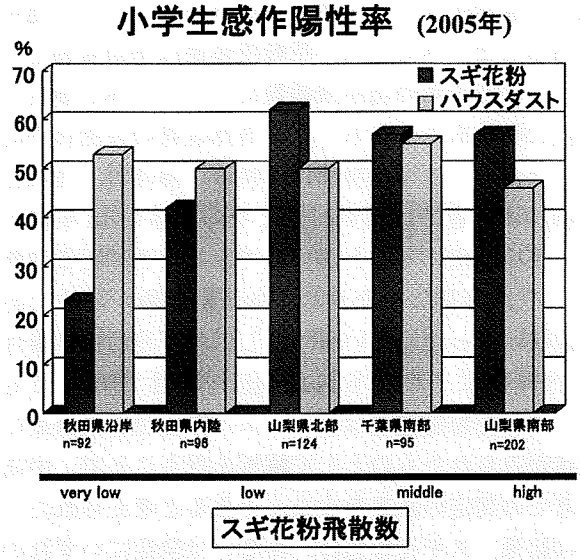


図4　2005年に行った小学生のスギ，ハウスダストに対する感作率の調査（文献9から）。

考えられている5箇所の農村部の小学校でアレルギー性鼻炎の検診を行った。すなわち，花粉飛散数が著しく少ない地区として秋田県大潟村（2005年のスギ花粉飛散数はシミュレーションから約500個/cm^2），比較的少ない地区として秋田県湯沢市（ヒノキ花粉がない，約2,000個/cm^2），山梨県北杜市（約2,200個/cm^2），やや多い地域として千葉県丸山地区（約4,000個/cm^2），非常に多い地域として山梨県南部町（約7,700個/cm^2）を選択し，可能であれば全校生徒，難しければ4，5年生全員を対象に行った。その結果，確かに花粉飛散数が非常に少ない大潟村での感作率は20%台と低く，花粉飛散数の増加とともに感作率は高くなる。しかし，飛散数がある程度多くなるといずれの地域でも感作率は50～60%とプラトーに達していた（図4）[9,10]。

特に山梨県の北杜市と南部町では毎年のスギ-ヒノキ花粉飛散数は3～4倍異なるが感作率にも発症率にも差が認められず，このことは感作率の増加と飛散花粉数にある程度の関連は認められるものの，他の要因の関与も大きく，たとえ花粉飛散量，曝露量を半分にしても一次介入，二次介入として必ずしも感作の予防や発症の予防につながらないことを示唆するものであった。抗原回避は意義があるものの，実際の介入としては机上で考えるほど容易ではないことを示すものと言える。

抗原特異的免疫療法（減感作療法）

スギ花粉に対する感作は小児期から成人期に年齢とともに増加し，また，多くが重複感作を受けていく。スギ花粉に対する感作率がダニに対する感作率を上回ってきていることから，ダニのみならずスギ花粉に対する感作が重複感作の第一段階と考えられる。抗原特異的免疫療法を受けた患者では重複感作を抑制することを示す可能性が報告されている[11,12]が，1970～1980年代にハウスダストによる免疫療法を受けた通年性アレルギー性鼻炎患者のその後のスギ花粉症の発症をみると免疫治療を受けなかった患者に比較して発症率が低い傾向にあった（表3）。有意差は認めなかったが，ハウスダストによる免疫治療を開始した時期も大きな要因になると考えられ，年少者でスギ花粉感作前に行うとその有効性は高いものと想定される。ただ，現行の皮下注射による免疫療法は頻回な通院が必要であり，稀とはいえ重篤な副作用の発現の可能性があること[13]から患者，医療機関への負担が大きく，特に年少者への頻回な注射接種は実際には困難である。

現在，舌下免疫療法が皮下注射に代わる投与法として注目され，国内でもスギ花粉症に対する開発が進んでいる（図5，6）[14～16]。今後，早期介入手段としても検討が進められるであろうが，実際にどの程度の重複感作の予防効果があるのかは国際的にも明

らかになっていない。今後の国内での検討が期待される。

薬物療法

アレルギー性鼻炎に対する一次介入，二次介入としての薬物療法の意義について検討は行われていないが，喘息発症の危険性が高いアトピー性皮膚炎の患者を対象に抗ヒスタミン薬の予防効果についてプラセボ対照に試験が行われている[17,18]。ただ，その結果は一致しておらず，国際的アレルギー性鼻炎のガイドラインとされるARIAにおいても，鼻炎に対する抗ヒスタミン薬の発症予防投与はエビデンスが不十分であり，副作用発現の危惧があることから推奨されていない[19]。

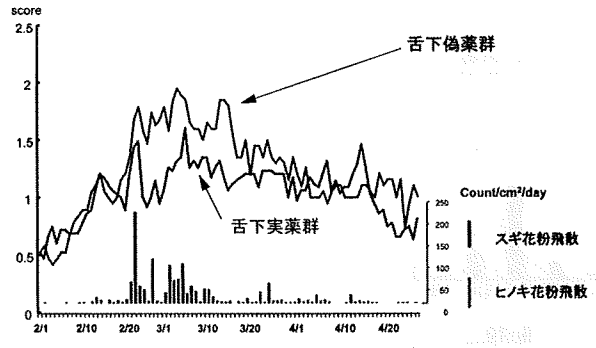


図5　スギ花粉症成人患者に対する半年間の舌下免疫療法のランダム化試験の効果（文献14から改編）。

花粉症においての薬物療法による初期治療が正に三次介入に相当すると考えられる。初期治療はスギ花粉症患者にスギ花粉飛散開始あるいはその直前，または飛散前であっても症状の発現がみられたら薬物治療を開始することで花粉飛散が増加し，ピークになっても鼻の過敏症状を改善することを期待するものである[18]。しかし，実際に初期治療に意義があるのか否かの評価はプラセボを初期治療薬の対照として同時期から投与を開始し，かつ飛散ピーク時の治療は症状の強さに基づいた標準治療（鼻噴射用ステロイド薬と抗ヒスタミン薬，あるいは抗ロイコトリエン薬の内服）を初期治療群と非初期治療群（プラセボ群）で同様に行い，その結果の検討からはじめて初期治療の有効性を評価することが可能となる。ただ，このような検討の実施はプラセボ薬の準備，試験の費用から容易ではなかったが，2007年に初めて実施することができた。その結果は，確かに抗ロイコトリエン薬を初期治療薬として投与した群では，プラセボ投与群に比較して花粉飛散ピーク時の症状スコアが低く（図7），さらに治療法の評価方法として鋭敏とされる患者のQOL評価ではより顕著に高い改善効果が認められ（表4），花粉症に対する初期治療の意義が確認された[20]。今後は，効果の持続性についても検討が必要であろう。

機能性食品

食品として用いられ，安全でかつ比較的安価であ

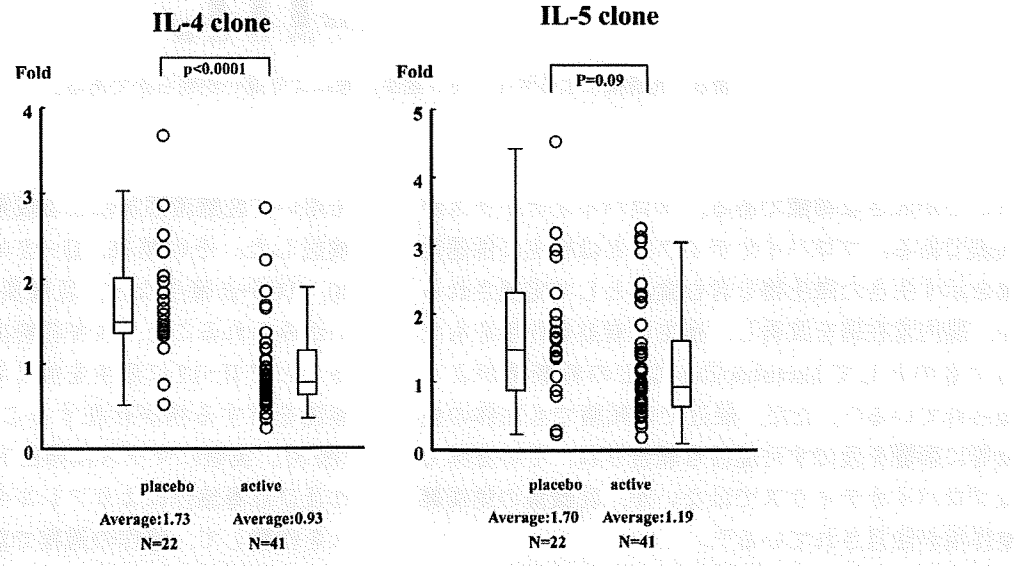


図6　スギ花粉舌下免疫療法により，スギCry jに対する特異的Th2サイトカイン産生クローンサイズの花粉飛散期の増加が抑制された（文献14から改編）。

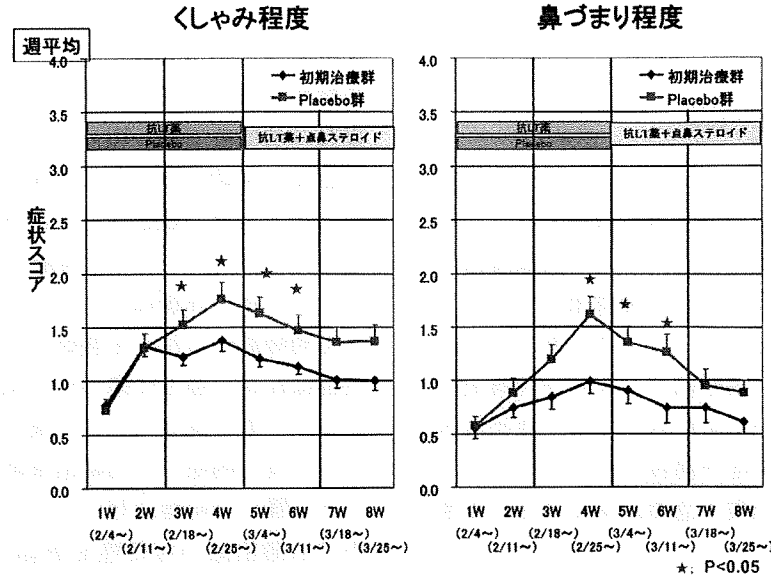


図7　抗ロイコトリエン薬を用いた初期治療の効果。プラセボ対照のランダム化試験（文献20から改編）。

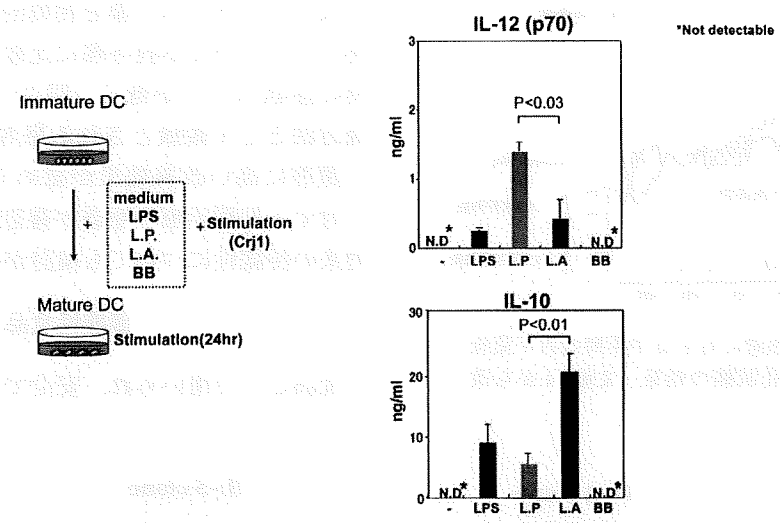


図8　乳酸菌によるサイトカン産生。株により違いが明らかである。

ることが大きな特徴である，プロバイオティクスが代表である。プロバイオティクスとは宿主に併速効果を示す生きた微生物を含む食品として定義されるが，腸内常在菌を改善し，宿主に有益な作用をもたらすものとしてLactobacillusなどの乳酸菌がよく知られている[21]。ただ，最近では死菌でも生体の免疫等に影響を及ぼす可能性も報告され，その意味ではプロバイオティクスではないが，乳酸菌の免疫調整作用が注目されている[22]。

乳酸菌（死菌）の樹状細胞に及ぼす影響を，スギ花粉症患者末梢血から分離，誘導した未熟樹状細胞を用いて乳酸菌添加による成熟樹状細胞への誘導で検討した。その結果，IL-12の産生，あるいはIL-10，TGF-β産生など，乳酸菌の株により大きな違いがみられること，スギ花粉抗原，Cry j 1を併用することでIL-12に産生を強く増加し，DC-1への誘導能を有する株が存在することが明らかになった（図8）。実際にマウスに卵白アルブミンを腹膜内感作及び点鼻感作によりアレルギー性鼻炎モデルマウスを作製して，感作の過程で乳酸菌（死菌）を経胃管投与の影響を検討すると，乳酸菌の摂取により，抗卵白アルブミンIgE抗体の有意な低下，抗原誘

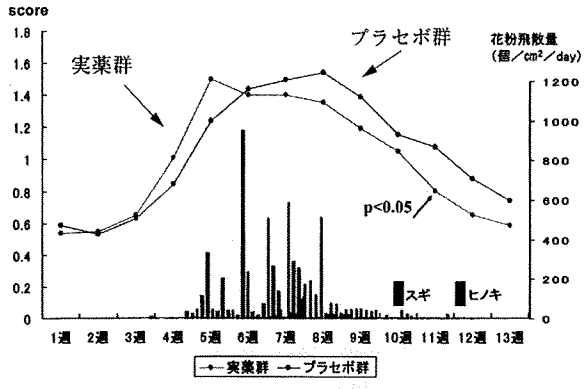


図9　スギ花粉症に対する乳酸菌の3次介入の効果
プラセボ対照のランダム化試験。

発による鼻症状の有意な改善が認められた。そこで，乳酸菌のIgE産生抑制効果，症状抑制効果を期待して，スギ花粉症患者，あるいはスギ花粉感作陽性だが未発症者，スギ花粉感作陰性かつ未発症者，スギ花粉感作陰性かつ未発症者を対象にスギ花粉飛散開始約8週前から，乳酸菌カプセルの連日投与をプラセボ対照に三次介入，二次介入，一次介入試験として行った。それぞれの介入試験の参加者は78名，40名，20名で一定の結論を得るには解析不十分な数であったが，単一施設でのpreliminaryな検討として実施した。しかし，結果は，一次介入，二次介入試験ではプラセボ群と差がみられず，有効性を確認できなかった。また，三次介入試験では，スギ-ヒノキ花粉飛散の後半にプラセボ投与群に比較して軽度の鼻症状の改善がみられたものの，標準治療の改善効果には見劣るものであった（図9）。マウスの実験で用いた乳酸菌の量の違い（5mg/匹/日　7日間，ヒトでは50mg/日，150日間）もあろうが，明らかにマウスの実験の限界を示す結果であった。現在は投与法を工夫して検討を進めているが，有用性の証明には慎重な解析の積み重ねが必要である。

おわりに

スギ花粉症の発症，重症化の阻止には早期介入が不可欠である。早期介入は特にスギ花粉症感作前，あるいはスギ花粉症発症前の小児が対象となることが多いが，当面の主な介入候補として，舌下免疫療法，プロバイオティクスを含む機能性食品が挙げられる。ただ，前述したように有効性，安全性について多くの慎重かつ科学的評価を進めていくことが必要である。三次介入としては薬物治療も期待されるが，効果の持続性についての検討も必要であることは言うまでもない。喘息での吸入ステロイドの投与は，中止後の効果の持続性が認められず否定的な結果が報告されている[23]。増加するスギ花粉症に対して早期介入の意義はあるが，標準化を進めるために，今後多くの質の高い臨床研究が必要とされている。

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Summary

Early Intervetions of Cedar Pollinosis

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In recent years, many countries have experienced an increase in the prevalence of allergic rhinitis. In Japan, Japanese cedar and cypress pollens constitute a major, unique allergen that's spread is quite large, traveling more than 100 km and causing pollinosis that is severer than observed in other countries. In addition, cedar and cypress pollen spread season lasts for more than 12 weeks in and around Tokyo. To manage allergic rhinitis, the interventions at various stages are important. The recent progress in genetic analysis revealed the presence of genes regulating IgE producing abilities. For early prevention of allergic rhinitis, the allergen avoidance is important, however it is not easy to get effective results. Drug treatment is useful to improve the symptoms, however the role in early intervention in allergic rhinitis is not known and to keep taking medicine for a long term may not be acceptable. Allergen specific immunotherapy is effective in early intervention, and has possibilities of usefulness in primary or secondary intervention. Probiotics may play some role in preventions of allergic rhinitis. Futher studies to certify and to improve the clinical benefits of these early interventions are required.

Key words：cedar pollinosis, early intervention, immunotherapy, medication, functional food

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Poly(I:C) induces BLyS-expression of airway fibroblasts through phosphatidylinositol 3-kinase

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ABSTRACT

B lymphocyte stimulator (BLyS), B cell activating factor (BAFF), a member of the tumor necrosis factor ligand superfamily has potent co-stimulatory activity on B cells, and BLyS-production in the airway mucosa is of potential importance as it triggers innate and adaptive immune responses. To investigate whether airway fibroblast could express BLyS, we examined BLyS-expression in human nasal airway fibroblasts and compared to its expression in tonsillar and skin fibroblasts as well as the effect of the Toll-like receptor (TLR) ligands on that in human nasal airway fibroblasts. The expression of BLyS by nasal fibroblasts in the presence of polyinocinic–polycytidykic acid (poly(I:C)) was markedly induced, to a level of more than 100 times higher than that observed in the absence of poly(I:C). In order to demonstrate the intracellular pathways involved in poly(I:C)-induced BLyS-expression, we used specific inhibitors of phosphatidylinositol 3-kinase (PI3-kinase), spleen tyrosine kinase (Syk), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), and extracellular-signal related kinase (ERK)-signaling in these events. Pre-incubation with the PI3-kinase inhibitor LY294002 or Wortmanin reversed the poly(I:C)-induced production and expression of BLyS. Syk kinase inhibitor Piceatannol partially reduced its production and expression. Thus, we were able to show that PI3-kinase signaling is directly involved in poly(I:C)-induced BLyS-expression in nasal airway fibroblasts. These results indicate that human nasal airway fibroblasts strongly induce BLyS-expression and production by poly(I:C) through PI3-K signaling during airway immune responses.

1. Introduction

TNF ligand superfamily member 13B, BLyS,[1] BAFF, plays critical roles in respiratory mucosal defense, because it is a potent co-activator of B cells in vitro or in vivo. BLyS induces B cell proliferation, human Ig class switch recombination, and Ig secretion [1–3]. Activation of B cells in the airways is now believed to be of great importance in immunity to pathogens, and it also participates in the pathogenesis of airway diseases. The expression of BLyS was detected in TLR ligand-treated BEAS-2B cells and primary human bronchial epithelial cells [4]. In the nasal mucosa, the expression of BLyS for in sinonasal tissue was found to be significantly correlated with CD20, and overproduction of BLyS contributes to the pathogenesis of chronic rhinosinusitis via the local induction of IgA and the activation of eosinophils [5]. Although nasal airway fibroblasts are a rich source of cytokines, chemokines, and growth factors, it is unknown whether airway fibroblast could express BLyS.

As 'a mucosal guardian' in the upper airway, the inferior turbinate of the human nose is easily exposed to a variety of stimulus such as viral and bacterial infection during the common cold. Most of the viruses that cause upper respiratory infection are RNA viruses including rhinoviruses, coxsackievirus, echovirus, and influenza viruses. RNA viruses synthesize double-stranded RNA (dsRNA) during replication [6], and this is a strong stimulus for innate anti-viral responses through the secretion of cytokines. TLRs play key roles in innate immunity by recognizing microbial conserved pathogen-associated molecular patterns, and TLR3 is involved in the recognition of the synthetic dsRNA analogue, polyinocinic–polycytidykic acid (polyI:C) [7]. It is not clear which TLR ligand induces BLyS-expression or production of human airway fibroblasts.

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[1] *Abbreviations used:* BLyS, B lymphocyte stimulator; BAFF, B cell activating factor; TLR, Toll-like receptor; dsRNA, double-stranded RNA; IRF, interferon regulatory factor; PGN, peptidoglycan; poly(I:C), polyinocinic-polycytidykic acid; LPS, Lipopolysaccharide; PI-3K, phosphatidylinositol 3-kinase; Syk, spleen tyrosine kinase; JNK, c-Jun N-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular-signal related kinase; TACI, transmembrane activator and CAML interactor.

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The presence of dsRNA during viral infections is a key step in the activation of several signaling pathways, including mitogen-activated protein kinase (MAPK), activator protein-1, and interferon regulatory factors (IRFs). Poly(I:C) induces the rapid activation of the mitogen-activated protein kinases (MAPKs) (p38 MAPK, c-Jun N-terminal kinase (JNK), ERK) and MAPK-dependent expression of proinflammatory cytokines, chemokines, and adhesion molecules [8,9]. In accordance with the expression of TLR3, poly(I:C) stimulation induces the activation of interferon regulatory factor-3 (IRF-3) transcription factor and p38 MAPK [10]. PI3-kinase plays an essential role in IRF-3-binding to the promoter of the target gene and TLR3-mediated gene induction by dsRNA-treated cells [11]. Spleen tyrosine kinase (Syk) regulates PI3K activation and RNA virus endocytosis in the airway mucosae [12]. The potential role of dsRNA-induced BLyS-expression in signaling is poorly understood.

Although the ability of BLyS-production from airway mucosa is of potential importance as it provides innate and adaptive immune responses, the details of BLyS-expresion in airway fibroblasts remains unexplored. In this study, we established fibroblast lines from the human inferior turbinate and other tissues, and it has been confirmed whether BLyS is expressed in human nasal airway fibroblasts, tonsillar, and skin fibroblasts. We examined the effect on BLyS-expression of TLR ligands including peptidoglycan (PGN); poly(I:C); lipopolysaccharide (LPS); and CpG in human nasal airway fibroblasts. In order to demonstrate the intracellular pathways involved in dsRNA-induced BLyS-expression, we used specific inhibitors of PI3-kinase, Syk, p38 MAPK, JNK, and extracellular-signal related kinase (ERK)-signaling in these events.

2. Materials and methods

2.1. Reagents

The following reagents were used: poly(I:C) (Amersham Bioscience, Piscataway, NJ); PGN (Sigma); LPS (MERCK bioscience, Germany); CpG, a synthetic oligodeoxynucleotide that contains CpG motifs that mimicks bacterial DNA (5′-ACCGATCGTTCGGCCGGT-GACGGCACCA-3′); SP600125 as a specific inhibitor of JNK (BIOMOL); SB203580 as a specific inhibitor of p38 MAP kinase (Promega); PD98059 as a specific inhibitor of MEK-1 (Promega); LY294002 as a specific inhibitor of PI3-kinase (Promega); Wortmannin (Sigma); AKT inhibitor (CALBIOCHEM); anti-human BLyS monoclonal Ab (R&D system); p44/42 MAP Kinase rabbit polyclonal antibody (Ab) (Cell Signaling, Beverly, MA); SAPK/JNK rabbit polyclonal Ab (Cell Signaling); phospho-p44/42 MAPK (E10) mouse monoclonal Ab (Cell Signaling); phospho-JNK (G9) mouse monoclonal Ab (Cell Signaling); phospho-p38 MAPK (28B10) mouse monoclonal Ab (Cell Signaling); phospho-AKT (587F11) mouse monoclonal Ab (Cell Signaling); AKT Rabbit polyclonal Ab (Cell Signaling); and p38 (A12) mouse monoclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

2.2. Cells, cell lines, and cell culture

Human primary nasal fibroblast lines were established from human nasal biopsy tissues of inferior turbinates removed during the operation ($n = 6$). All the nasal specimens had been taken from patients with allergic rhinitis. Five males and one female aged 30.5 ± 6.6 year (mean ± SEM) were atopic, diagnosed on the basis of elevation of at least one of the capsulated hydrophobic carrier polymer-radioallergosorbent tests against 8 common aeroallergens. All of the patients had a house dust or a cedar pollen CAP-RAST score of 2 or more. The subjects had given written informed consent, and its study protocol was approved by the Ethics Committee at University of Fukui. The patients had no smoking and no special background including pollution, without any medication at least 14 days before operation. Only fibroblast lines between the sixth and tenth passages were used in this study. No contamination of epithelial cells was confirmed by immunohistochemical examination using cytokeratin markers. The fibroblasts were stimulated by TLR ligands in RPMI-1640 medium supplemented with 10% FCS and in humidified atmosphere of 10% CO_2 in air at 37 °C.

2.3. Real time PCR

Total RNA was extracted using a total RNA isolation NucleoSpin™ RNA II Kit (MACHERY-NAGEL, Düren Germany). The reverse transcription reaction was performed with TaqMan® RT Reagents (Applied Biosystems Japan, Tokyo, Japan) using random hexamer primers. The amplification of TLRs, BLyS, and β_2-microglobulin-cDNA was performed in a MicroAmp optical 96-well reaction plate (Applied Biosystems). All TaqMan® probe/primer combinations used in this study were TaqMan® Gene Expression Assay products purchased from Applied Biosystems. β2-Microglobulin was chosen as the reference housekeeping gene because it is convenient to assay and highly expressed. Furthermore, in order to select the housekeeping gene, we evaluated it using a TaqMan® Human Endogenous Control Plate, which was most suitable. TaqMan® PCR was performed in a 20-μl volume using TaqMan® Universal PCR master mix (Applied Biosystems). The reaction was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The reaction mixtures were pre-incubated for 2 min at 50 °C. The PCR program involved 10 min of Taq Gold activation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C (maximum ramping speed between temperatures). Human cDNA equivalent to 50 ng of total RNA from each sample was assayed in each tube. The threshold cycle number was determined with sequence Detector Software (version 1.1: Applied Biosystems) and transformed using comparative methods as described by the manufacturer with β_2-microglobulin as the reference gene.

2.4. Cytokine assay

The cells were cultured in the presence of poly(I:C) for appropriate periods, and then the culture supernatants were harvested and stored at −80 °C. The amounts of BLyS in the cell culture supernatant were measured with an ELISA kit that was purchased from R&D system.

2.5. Immunoblot analysis

The samples were added to a 2-fold volume of sample buffer [95% laemmli sample buffer (BIORAD) and 5% 2-mercaptoethanol]. After heating the mixture at 95 °C for 5 min, the samples were electrophoresed. The proteins were then transferred electrophoretically onto polyvinylamidedifluoride membranes (Amersham Bioscience). The blotted membranes were rinsed with 5% non-fat-dried milk diluted in PBS containing 0.1% Tween 20 for 60 min at room temperature, and then incubated with the antibodies for 16 h at 4 °C. After being washed, the membranes were treated with HRP-conjugated anti-mouse immunoglobulin (Ig) Ab or HRP anti-rabbit Ig Ab (DAKO, Carpinteria, CA) for 60 min at room temperature. Peroxidase color visualization was achieved with TMB membrane peroxidase substrate (KPL, Gaithersburg, MD).

2.6. Antibody array

Signal Transduction AntibodyArray™ which contains 400 high quality antibodies against well-studied signaling proteins, was purchased from Proteomics Company. Nasal fibroblasts were stim-

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ulated with 10 μg/ml poly(I:C) for 30 min, washed twice with ice cold Tris saline (50 mM Tris pH 7.5, 150 mM NaCl, 1.5 mM PMSF), and lysed using Triton Extraction buffer containing 15 mM Tris pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT, 0.5% triton X-100, 10 μg/ml leupeptin, and 0.5 mM PMSF. Pelleted cellular debris was removed by centrifugation at maximum speed (14,000 rpm). The supernatant was collected, and the membrane of Signal Transduction Antibody Array™ was incubated with the whole cell extracts in 5 ml extraction solution containing 1% BSA for 2 h at room temperature with slow shaking. After washing the membrane, HRP-conjugated anti phosphotyrosine antibody was applied for 2 h at room temperature. Peroxidase substrate was used and the membrane was washed and then exposed to X-ray film.

2.7. Data and statistical analysis

Statistical analysis was performed using the Wilcoxon signed-ranks test to assess the significance of differences.

3. Results

3.1. BLyS-expression in human fibroblasts

To determine whether BLyS is expressed in human fibroblasts, we established fibroblast lines from small pieces of human inferior turbinate, tonsil, and skin respectively from six individuals and then examined BLyS-expression in stimulated fibroblasts. As shown in Fig. 1, the expression of BLyS in nasal fibroblasts was markedly induced in the presence of poly(I:C), to a level more than 100 times higher than that observed in the absence of poly(I:C). In skin fibroblasts, we could not detect any induction of BLyS-expression in the presence of poly(I:C). Although poly(I:C) induced BLyS-expression by tonsillar fibroblasts, its induction was lower than that induced in nasal stimulated fibroblasts .

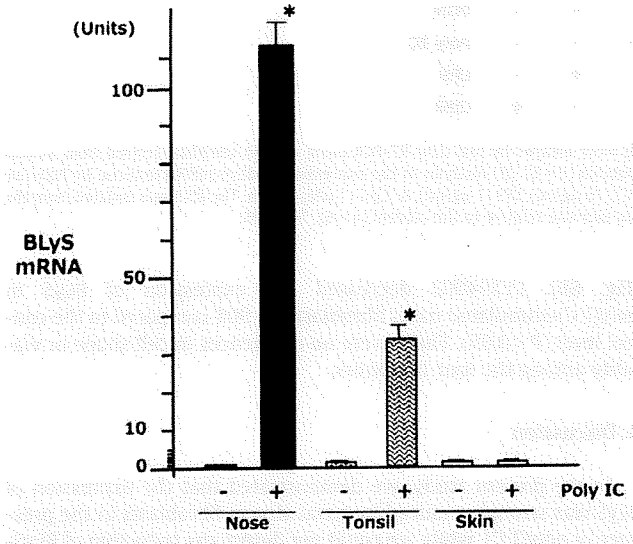

Fig. 1. BLyS-expression in human fibroblasts. After the cells has been treated with poly(I:C) (20 μg/ml) for 6 h, total RNA was isolated from human nasal (closed bar), tonsillar (waved bar), and skin (open bar) fibroblasts. The expression levels of BLyS-mRNA were assayed by real time RT-PCR. The RNA was reverse transcribed to cDNA, which was then used for real time PCR. Reactions were performed in three wells, and results are expressed relative to the expression levels of β_2-microglobulin. Data are expressed as the mean ± SEM of the fold increase relative to the control ($n = 6$). *$P < 0.05$ compared with control using Wilcoxon's signed-ranks test.

3.2. Toll-like receptor ligands and BLyS-expression

Since TLR 3 ligand strongly induced the expression of BLyS especially in human nasal fibroblasts, next we observed the expression of TLRs on human nasal fibroblasts. The mRNA expression of TLRs on fibroblasts was confirmed by real time RT-PCR. Fig. 2A shows the relative expression levels of TLR mRNAs on the cells. TLR 3 and 4 were highly expressed, while TLR 2 and 9 were moderately expressed. TLR1, 5, and 6 were also detected, but their expression levels were lower than those of TLR2, 3, 4, and 9. We could not detect the expression of TLR 7, 8, and 10 in human nasal fibroblasts. In order to look at which TLR ligand induces BLyS-expression in human nasal fibroblasts, we examined the effect on BLyS-expression of TLR ligands (PGN, poly(I:C), LPS, and CpG). Fibroblasts were treated with the agonists for 6 h, and BLyS-mRNA expression was assessed by real time PCR. The BLyS-expression was induced 100-fold by poly(I:C) and 10-fold ($P < 0.05$) by LPS in nasal fibroblasts, while it was hard to find any effecet by PGN or CpG on the level of mRNA for BLyS.

3.3. Dose-dependence and time-course of poly(I:C)-induced BLyS-expression

Having shown that TLR3 ligands strongly induce BLyS-expression in human nasal fibroblasts, next we have investigated its expression precisely. Poly(I:C) induced BLyS-mRNA expression in a dose-dependent manner with the maximal stimulation generally being at 10 μg/ml or higher, and its expression was detected 10-fold ($P < 0.05$) at 1 μg/ml in nasal fibroblasts (Fig. 3A). The exposure of nasal fibroblasts to TLR3 ligand triggered a rapid expression of BLyS-mRNA at 6 h and decreased thereafter. The expression was sustained at 80-fold at 24 h and 15-fold ($P < 0.05$) at 48 h (Fig. 3B).

3.4. Poly(I:C) induces BLyS-production from human nasal fibroblasts

Similar to other TNF family members, BLyS is generally expressed as a transmembrane protein and is cleaved from the surface to release its active soluble form. Production of soluble BLyS-proteins was detected using ELISA. Poly(I:C) increased BLyS-production at 1 μg/ml and higher ($P < 0.05$) in a dose-dependent manner (Fig. 4A) . Its production was 100 times higher than that detected in the absence of poly(I:C) at 10 μg/ml. Although the BLyS gene encodes a putative 285 amino acid (aa) type II transmembrane protein, the 152 aa form can also be shed from the membrane because the N-terminal side contains a furin cleavage site. We also examined the supernatants from human nasal fibroblasts using Western blotting. Fig. 4B shows that the soluble form of BLyS from human nasal fibroblast weighs 18 kDa, and its production occurred in a dose-dependent manner with the maximal stimulation observed at 10 μg/ml.

In the presence of IL-4, Ig class switch recombination in human B cells was always detected by BLyS-treatment at 100 ng/ml or higher [2]. The human nasal fibroblasts produce enough amounts of BLyS-protein to cause Ig class switch recombination, as shown in Fig. 4.

3.5. Suppression of poly(I:C)-induced and BLyS-production and expression by PI3-kinase inhibitor

Poly(I:C) is a TLR3 ligand that induces TLR3-signaling. We screened the intracellular signal transduction molecules of poly(I:C)-stimulated human nasal fibroblasts using *AntibodyArray*™ which contains 400 high quality antibodies, and found that poly(I:C)-induced signaling involved Syk, Rho, or TRAF6. Also, we demonstrated that the exposure of cells to poly(I:C) triggered phosphorylation and activation of p38 MAPK, JNK, and AKT by

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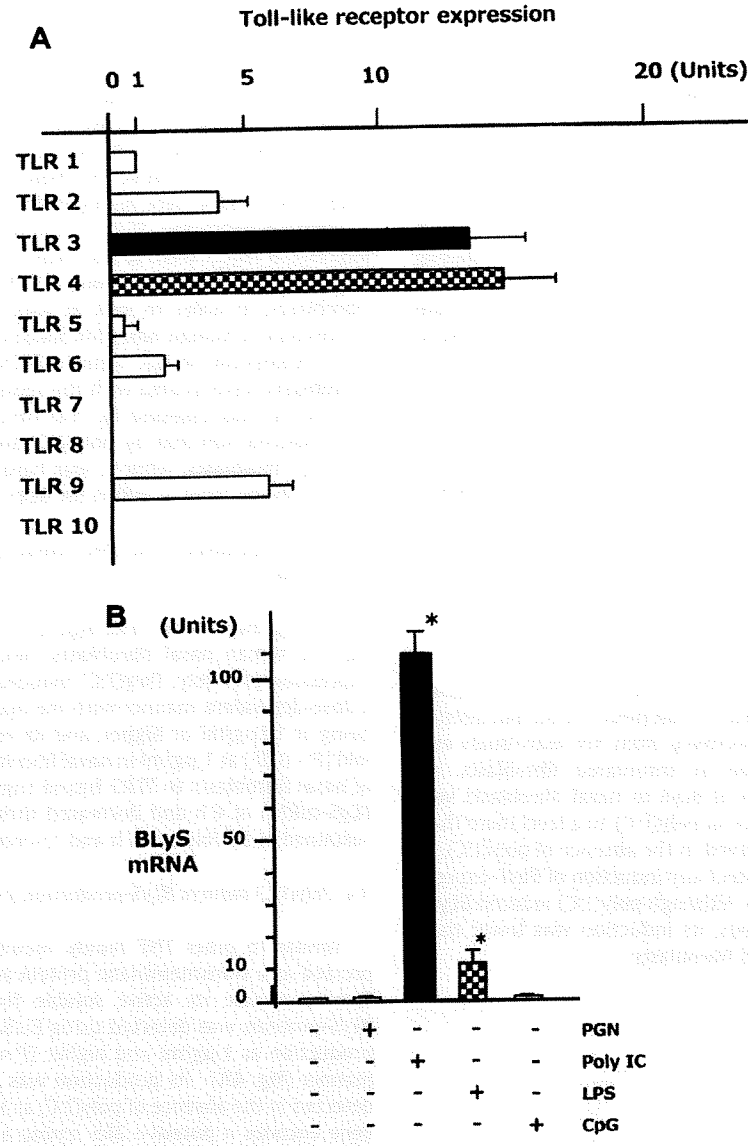

Fig. 2. Toll-like receptor ligands and BLyS-expression. (A) TLR1–9 mRNA expression levels were assayed by real time RT-PCR using a cDNA template derived from human nasal fibroblasts. The results are expressed relative to the expression levels of β_2-microglobulin ($n = 6$). (B) Analysis of the expression of BLyS-mRNA induced by Toll-like receptor ligands using real time PCR. Fibroblasts were treated with PGN (20 μg/ml), poly(I:C) (20 μg/ml), LPS (1 μg/ml), or CpG (1 μM) for 6 h. The RNA was isolated from the cells and BLyS-expression was measured. Data are expressed as the mean ± SEM of the fold increase relative to the control ($n = 6$). *$P < 0.05$.

Western blotting with specific antibodies to phosphorylated p38 MAPK, JNK, and AKT. Since PI3-kinase participates in TLR3-signaling [11], we sought to determine whether MAPKs, Syk kinase, or PI3-kinase signaling is directly involved in BLyS-expression. To accomplish this, we tested the ability of SB203580 (a specific inhibitor of p38 MAPK signaling), SP600125 (JNK inhibitor), PD98059 (a specific inhibitor of ERK signaling), Piceatannol (an inhibitor of Syk kinase), LY294002 and Wortmanin (PI3-kinase inhibitor) to affect the expression of BLyS in nasal fibroblasts stimulated with poly(I:C) (Figs. 5 and 6A).

Pre-incubation with the PI3-kinase inhibitor LY294002 and Wortmanin markedly suppressed the poly(I:C)-induced production and expression of BLyS, although inhibition of p38 MAP, JNK, and ERK did not have any effect. The Syk kinase inhibitor Piceatannol reduced poly(I:C)-induced production and expression of BLyS by 40% (Figs. 5 and 6A). The specific PI3-kinase inhibitor LY294002 reversed its expression in a dose-dependent manner (Fig. 6B). LY294002 decreased the expression of BLyS in poly(I:C)-stimulated nasal fibroblasts by 90% compared to the control level ($P < 0.05$). There were no differences in cell shape or viability among the four inhibitors.

4. Discussion

In the present study, we demonstrated that the expression of BLyS was strongly induced by nasal airway fibroblasts in the presence of poly(I:C), while we could not detect any induction of BLyS-expression in skin fibroblasts. Consistent with the high expression of TLR 3 and 4 mRNA on nasal airway fibroblasts, poly(I:C) and LPS induced BLyS-expression. Poly(I:C) induced BLyS-mRNA expression and protein-production in a dose-dependent manner. BLyS-expression and production from airway fibroblasts has not been reported previously, although BLyS is expressed in proinflammatory-cytokine-stimulated fibroblast-like synoviocytes from the inflamed

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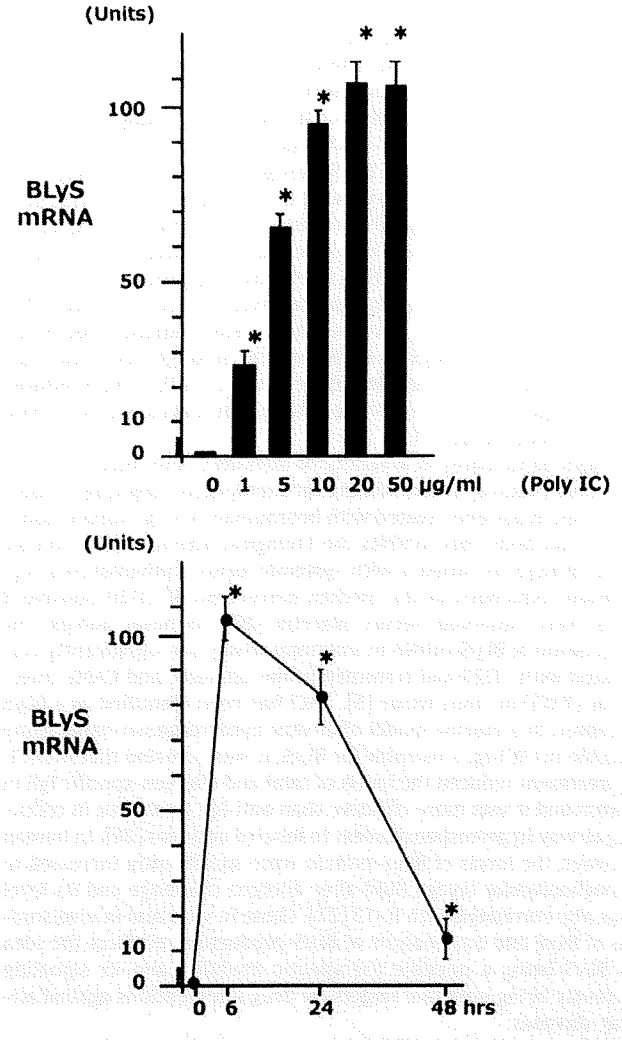

Fig. 3. Dose-dependence and time-course of poly(I:C)-induced BLyS-expression. (A) Human nasal fibroblast cells were cultured either with medium or with various concentrations of poly(I:C) (1, 5, 10, 20, and 50 μg/ml) for 6 h. (B) The cells were stimulated with poly(I:C) (10 μg/ml) and then harvested at 6, 24, 48, or 72 h. RNA was prepared and reverse transcribed to cDNA, followed by real time PCR. Data are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$.

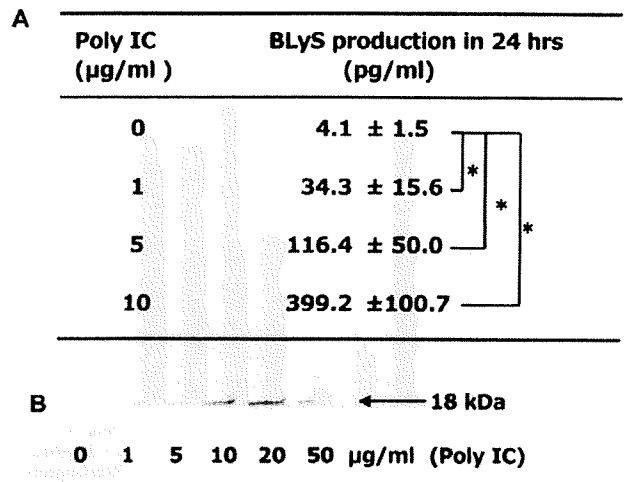

Fig. 4. Poly(I:C) induces BLyS-production from human nasal fibroblasts. (A) The cells were cultured either with medium or with various concentrations of poly(I:C) for 24 h. The supernatants were harvested for analysis of BLyS-production by ELISA. The results are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$. (B) The same amount of supernatant was applied to each lane and blotted with anti-BLyS Ab. The position of BLyS-proteins was indicated to the right with the arrow.

Inhibitor (10 μM)	Poly IC (μg/ml)	BLyS production in 24 hrs (pg/ml)
None	0	4.1 ± 4.4
None	10	391.8 ± 72.7
LY 294002	10	13.3 ± 5.9
Piceatannol	10	245.1 ± 52.8
SB203580	10	279.9 ± 51.2
PD98059	10	322.9 ± 64.9
SP600125	10	296.5 ± 82.3

Fig. 5. The effect of intracellular signal transduction inhibitors on Poly(I:C)-induced BLyS-production from human nasal fibroblasts. After pre-incubation with LY294002, Wortmanin, an inhibitor of PI3-kinase; Piceatannol, an inhibitor of Syk kinase; SB203580, an inhibitor of p38 MAP kinase; PD98059, an inhibitor of MEK; or SP600125, an inhibitor of JNK, the cells were stimulated with poly(I:C) (1, 5, and 10 μg/ml) for 24 h. The supernatants were harvested for analysis of BLyS-production by ELISA. The results are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$.

joints of patients with rheumatoid arthritis [13]. Pre-incubation with the PI3-kinase inhibitor LY294002 or Wortmanin reversed the poly(I:C)-induced production and expression of BLyS. The Syk kinase inhibitor Piceatannol also partially reduced its production and expression. Thus, we were able to show that PI3-kinase signaling is directly involved in poly(I:C)-induced BLyS-expression in nasal airway fibroblasts.

Airway submucosal fibroblasts play important roles, in the innate anti-viral response to human rhinovirus infections and dsRNA [14], while airway fibroblasts support the proliferation of bronchial epithelial cells, which is an important biological process in physiological conditions and various human airway diseases [15]. The inferior turbinate of the human nose is a 'mucosal protector' on the upper airway that acts against viral or bacterial infection and antigen exposure. Nasal fibroblasts of the inferior turbinate produce BLyS strongly when stimulated by dsRNA, leading to the innate anti-viral response including B cell proliferation and Ig secretion. Here, we have demonstrated the high BLyS-expression of nasal fibroblasts originating from nasal mucosa of the inferior turbinate.

Upper airways characteristically consist of a periosteum and bone covered by nasal respiratory mucosa. We also examined BLyS-expression of nasal fibroblasts originating from the periosteum of the inferior turbinate, and poly(I:C) strongly also induced BLyS-expression in these cells (data not shown). Fibroblasts also play key roles in airway remodeling process [16,17] and in nasal polyps, which are products of nasal airway remodeling, BLyS-mRNA was significantly increased in nasal polyps of patients with chronic rhinosinusitis, and its protein was present in mucosal epithelial cells in the nasal polyps along with unidentified cells in the lamina propria[5]. We also found that nasal fibroblasts originating from nasal polyps express BLyS by stimulation with dsRNA and LPS (data not shown).

Due to the high expression of TLR3 and TKR4, nasal fibroblasts can respond to their ligands. Through TLR3 and adaptor molecules, poly(I:C) stimulation induces the activation of IRF-3 transcription factor [10]. When PI3-kinase is not recruited to TLR3 or its activity

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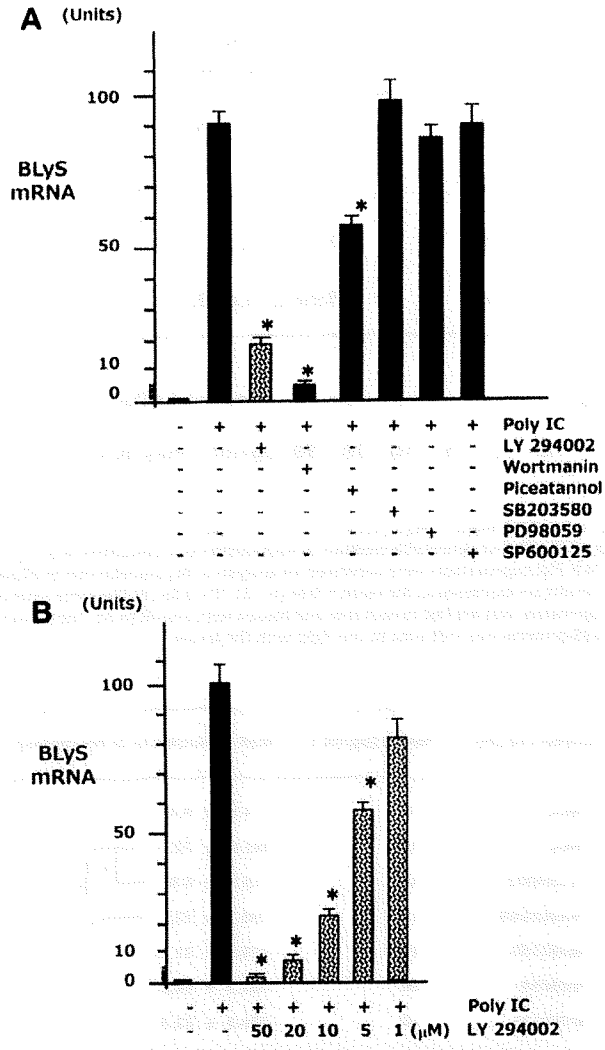

Fig. 6. Suppression of poly(I:C)-induced BLyS-expression by a PI3-kinase inhibitor. (A) Human nasal fibroblasts were pre-incubated with LY294002, Wortmanin, Piceatannol, SB203580, PD98059, or SP600125. The cells were harvested 6 h after stimulation with poly(I:C) (10 μg/ml), and the RNA was prepared for analysis of BLyS-expression by RT-PCR. Data are presented as mean ± SEM compared with the levels without inhibitor using Wilcoxon's signed-ranks test ($n = 6$). $^*P < 0.05$. (B) Dose response of PI3-kinase inhibition of poly(I:C)-induced BLyS-expression. The culture conditions were the same as those for the experiment shown in Fig. 5A. except that the concentration of LY294002 was varied as indicated.

is blocked, IRF-3 is only partially phosphorylated and fails to bind to the promoter of its target gene in dsRNA-treated cells [11]. The PI3-kinase pathway plays an essential role in TLR3-mediated gene induction. As a specific PI3-kinase inhibitor reversed the expression of BLyS in dsRNA-stimulated nasal fibroblasts, PI3-kinase is considered to play a pivotal role in dsRNA-induced BLyS-expression in human nasal fibroblasts. PI3-kinase inhibitor also decreased TLR3-experssion in nasal fibroblasts (data not shown). A frequent byproduct of virus infection, dsRNA, is recognized by TLR3 as a method for mediating the innate immune response to virus infection. The most common acute infection in humans, human rhinovirus is a leading cause of exacerbations of airway inflammation. We previously reported that the protein tyrosine kinase Syk is expresses in nasal fibroblasts [18,19] and regulates PI3K activation and human rhinovirus endocytosis [20]. Although the full intracellular signaling pathway for dsRNA-induced BLyS-expression remains to be elucidated, we did find that, a PI3-kinase inhibitor and a Syk kinase inhibitor significantly reduced dsRNA-induced production and expression of BLyS in nasal fibroblasts.

While BLyS is an important survival factor for B lymphocytes, it can enhance immune responses not only by increasing the number of B cells but also by elevating CD4-positive T lymphocyte function and NK cell activity [21]. In BLyS-transgenic mice, the delayed-type hypersensitivity scores were found to correlate directly with BLyS levels in serum [22]. BLyS also provides a co-stimulatory signal to T cells and T cell activation, and bronchial structural cells including fibroblasts might play a critical role in the regulation of inflammation in asthma by increasing the survival of T lymphocytes [23]. Human post-switched IgG-positive B cells respond specifically and exclusively to BLyS by differentiating into IgG-secreting plasma cells [24]. On the contrary, in the presence of IL-4, BLyS induced immunoglobulin class switch recombination to epsilon in a CD40-independent manner [1,2].

BLyS participates in a variety of disorders, and interruption of the BLyS pathway is a candidate for therapeutic targeting of some diseases. In patients treated with belimumab, a fully human monoclonal antibody that inhibits the biological activity of the soluble form of BLyS in patients with systemic lupus erythematosus, significant reductions in the median percentage of CD20 positive B cells were observed versus placebo [25]. In nasal polyps, the expression of BLyS-mRNA in sinonasal tissue was significantly correlated with CD20 and transmembrane activator and CAML interactor (TACI) in sinus tissue [5]. TACI has been identified as a BLyS receptor. In a murine model of airway hyperresponsiveness, using soluble mTACI-Ig, a receptor for BLyS, it was revealed that mTACI-Ig treatment reduced the levels of total and allergen-specific IgE in serum and it was more effective than anti-IgE treatment in reducing airway hyperresponsiveness to inhaled antigens [26]. In human airways, the levels of BLyS-protein were significantly increased in bronchoalveolar lavage fluid after allergen challenge and its level was also correlated with IL-13 [27]. These in vitro and in vivo studies of BLyS and our analysis of BLyS-production reinforce the idea of BLyS being a possible therapeutic modality and its signaling pathway being potential targets for drug interventions against airway diseases.

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RHINOLOGY

Bax-gene transfer enhances apoptosis by steroid treatment in human nasal fibroblasts

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Abstract Gene therapy has become a focus not only in the study of cancer but also lifestyle-related diseases. In case of chronic rhinosinusitis with nasal polyps and aspirin-induced asthma, nasal polyps poorly respond to a local administration of steroid. The Bax and Bcl-2 proteins play important roles in the regulation of apoptosis. The treatment of steroid (prednisone) induced apoptosis in the fibroblast. The Bax accelerates apoptosis. Apoptosis is very important in the anti-inflammatory mechanism. In this study, we investigated whether the overexpression of Bax in human fibroblasts influences apoptosis by treatment with a steroid (prednisolone) in vitro. Human nasal fibroblasts were isolated from small pieces of nasal polyp and were transfected with a *bax* gene-bearing mammalian expression vector. Human nasal fibroblasts were transiently transfected with the expression vector hBaxpcDNA3 (Bax-NF) or native pcDNA3 (Neo-NF). Both transfectants (Bax-NF, Neo-NF) and wild-type-nasal fibroblast (wt-NF) were cultured in conditioning medium and treated with each concentration of prednisolone for 72 h. Prednisolone at a concentration of 10 ng/ml decreased the viability of Bax-NF compared to that of Bax-NF in the absence of prednisolone. The cytotoxicity of prednisolone to Bax-NF was significantly higher than that to Neo-NF or wt-NF ($p < 0.01$) and the susceptibility of Bax-NF to prednisolone was about 1,000 times that of Neo-NF or wt-NF. We found that the transfer of the exogenous *bax* gene enhanced the induction of apoptosis by steroid-treatment in human nasal fibroblasts. Therefore, we suggest that exogenous Bax protein expression by gene transfer might be useful for the treatment of nasal polyps. We will further the preclinical study in improving steroids dose and in adopting to transfer bax gene to the nasal polyps by intranasal injection, thus providing a more effective and safer way for the nasal polyps that poorly respond to a local administration of steroids.

Keywords Bax overexpression · Nasal polyp · Prednisolone · Gene therapy · Chronic sinusitis · Glucocorticoid

Introduction

Nasal polyps are a chronic inflammatory disease of the upper airway, leading to recurrent protrusions of benign edematous nasal mucosae from the nasal sinuses into the nasal cavities. Nasal polyps are not a simple edema of the mucous membrane. They are characterized by the infiltration of inflammatory cells, such as eosinophils, basophiles, and lymphocytes and structural abnormalities including stromal fibrosis. Fibroblasts are resident cells thought to effect the development of fibrosis. The proliferation of stromal and epithelial elements is thought to account for the growth of nasal polyps [1]. It is proposed that fibroblasts play a critical role in the switch from acute inflammation to adaptive immunity and tissue repair. Fibroblasts actively define the structure of tissue microenvironments and modulate immune cell behavior by conditioning the local cellular

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and cytokine microenvironment so that the kinetics and nature of the inflammatory infiltrate are appropriate to the cause of the damage [2]. Nasal polyp-derived fibroblasts produce a large amount of RANTES by IFN γ (Th1 cytokine) [3] and eotaxin by IL-4 (Th2 cytokine) to attract and activate eosinophils [4]. Nasal fibroblasts also spontaneously release IL-8 and GM-CSF, and enhanced IL-8 production by the inflammatory cytokine IL-1 band TNFa induces neutrophils to move into the infected area [5].

Apoptosis, which is regulated both by cell survival and by death signals, is important for the swift clearance of unwanted cells. The treatment of nasal polyps involves an important role for apoptosis in the removal of inflammatory cells and resolution of inflammatory processes. Glucocorticoids have been shown to induce apoptosis in most nucleated cells, such as thymocytes, myeloma cells, and peripheral blood monocytes and play a major role in the attenuation of inflammatory responses [6, 7]. Topical steroids have been demonstrated to be effective in the treatment of nasal polyps [8]. The administration of topical steroids in vivo increased apoptotic index of eosinophils and T-lymphocytes in nasal polyps [9]. After oral prednisolone treatment, eosinophilic apoptosis was accompanied by a significant decrease in the number of EG2 and IL-5 double positive cells [10]. Treatment with glucocorticoids in vitro increased the apoptosis of both eosinophils and T-lymphocytes derived from nasal polyps in a specific culture system [9]. Also, glucocorticoids induced the apoptosis of nasal polyp-derived fibroblasts [11] and stromal cells [12].

Induction of apoptosis by gene transfer into appropriate target cells and tissues has become a focus of study as a therapeutic approach of cancer [13, 14]. The Bcl-2/Bax system and Fas/Fas-ligand system play a crucial role in the regulation of the apoptotic process. Bcl-2 gene was the first to be identified because of its involvement in the *t*(14, 18) chromosomal translocation found in many B-cell lymphomas. Bax is a member of an apoptosis-promoting protein and has an antagonistic role against the action of Bcl-2. Both Bcl-2 and Bax proteins would therefore be important in the regulation of apoptosis. Bcl-2 is an intercellular protein that inhibits apoptosis while Bax counteracts the anti-apoptotic function of Bcl-2 by binding to this molecule [15].

Although glucocorticoids have been widely employed for the treatment of nasal polyps and allergic sinusitis, serious problems include poor response or resistance to steroids in patients with aspirin-induced asthma. In this study, we investigated whether a *bax* gene transfer to human fibroblasts treated with prednisolone would influence the induction of apoptosis to establish a more effective steroid therapy for nasal polyps.

Materials and methods

Cell lines and culture conditions

Nasal polyps were obtained from patients with chronic rhinosinusitis with nasal polyp during endonasal sinus surgery. Aspirin-induced asthma was excluded. Nasal specimens were cultured in 10-cm dishes containing RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated FCS (Gibco, Grabd Island, NY), 0.29 mg/ml glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin, at 37°C in 5% CO_2 humidified air. After a period of 3 to 4 weeks, when the growth of nasal fibroblasts was established, nasal fragments were removed and the first passage was performed [3, 16]. The cells were used at passage number 3 to 5. There was no contamination by epithelial cells as confirmed by immunohistochemical examination using a cytokeratin marker.

Informed consent for surgery and participation in the study was obtained from all patients and their families.

Transfection

The expression vector hBaxpcDNA3 was used with the permission of Drs. J.-C. Martinou and R. Brown (Glaxo Wellcome, Bedsurham, UK). This vector containing a full-length human *bax* cDNA tagged at the 3′ with 57 bps was subcloned into the mammalian expression pcDNA3 (Invitrogen, Carlsbad, CA) to distinguish the transfected tagged-Bax protein from intrinsic Bax protein [14]. Bax-nasal fibroblasts (Bax-NF) were transiently transfected with hBaxpcDNA3. Neo-NF was transiently transfected with native pcDNA3. Both vectors were introduced into nasal fibroblasts using the effectene transfection reagent (QIAGEN, Valencia, CA) under the conditions recommended by the manufacturer.

MTT assay

Prednisolone (1 mg) was dissolved in DMSO (5 ml, Wako, Osaka, Japan) and diluted with phosphate-buffered saline. Approximately 5×10^3 cells were seeded in each well of a 96-well plate in triplicate and cultured in 200 µl of medium. After 24 h, 1 µl of each concentration of prednisolone solution was added to the well, and the cells were cultured for 72 h. At that stage, the medium was removed, 10 µl of 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) in PBS was administered and the cells were incubated at 37°C for 4 h. DMSO (200 µl) was added and the absorbance of each well was measured at 540 nm (reference absorbance at 630 nm). All values represent the mean ± standard deviation (SD) from triplicate cultures. The test was performed independently 6

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