BACKGROUND: The activation of signal transducer and activator of transcription (STAT) protein is thought to lead to the genesis of neoplasia by promoting cellular growth and preventing apoptosis, and by immune system modulation; STAT3 protein has also been implicated in tumor survival and propagation in recurrent respiratory papillomatosis (RRP).

OBJECTIVES: To investigate the presence and activation of STAT3 protein in papilloma specimens from subjects with RRP and compare findings with those obtained from control subjects’ tissue.

DESIGN: Laryngeal papilloma samples were collected from 8 nonselected consecutive patients undergoing surgery for RRP, and control samples of anterior tonsillar pillar mucosa were collected from 8 patients undergoing adenotonsillectomy. After extraction, we applied gel shifting to the nuclear protein using an electromotility shift assay kit. Quantitative analysis of the gel shifts was performed, and levels of activated STAT3 protein in RRP specimens and tissue from controls were compared.

RESULTS: There was STAT3 protein activation in the nuclear extracts of all (100%) RRP specimens, which was significantly more frequent than in normal epithelial tissue from controls (P<.03).

CONCLUSIONS: We conclude that STAT3 protein activation is present in RRP. However, further study is needed to determine if STAT3 protein activation is an important pathway through which human papillomavirus results in the propagation and persistence of RRP.


METHODS

Papilloma samples were collected from 8 patients with histologically confirmed RRP without atypia. Normal (control) specimens were obtained from the anterior tonsillar pillar of 8 patients undergoing adenotonsillectomy. These tissues were obtained in compliance with institutional human subjects research guidelines, and approval for this study was granted.

©2004 American Medical Association. All rights reserved.
by the Children's Hospital of Wisconsin institutional review board. The tissue collected was immediately placed in liquid nitrogen. For nuclear protein extraction a standard protocol (Active Motif, Carlsbad, Calif) and for gel shift assays the EMSA Kit (Panomics, Redwood City, Calif) were used. Western blot assays confirmed that the STAT proteins in the nuclear extracts were activated phospho-STAT3 proteins. Histogram analysis of each gel shift was performed with Adobe Photoshop software (Adobe Systems Inc, San Jose, Calif), and statistical analysis was performed using the paired t test.

**NUCLEAR EXTRACTION**

The acquired tissue was homogenized and suspended in a hypotonic buffer solution consisting of HEPES, magnesium chloride, potassium chloride, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and deionized water on ice. The tissue was allowed to swell and lyse for 10 minutes. After centrifuging the mixture at 500 rpm for 5 minutes, the supernatant consisting of cytoplasmic extract was discarded. The remaining nuclear pellet was suspended in a hypertonic solution consisting of HEPES, glycerol, sodium chloride, magnesium chloride, EDTA, DTT, PMSF, and deionized water. The solution was centrifuged at 14,000g for 10 minutes, and the supernatant nuclear extract was aliquoted and stored at −83°C until analyzed by gel shift assay.

**GEL SHIFTS**

Eight micrograms of nuclear extract were loaded into wells of 6% polyacrylamide gel with 5 × binding buffer, Poly (dl-C), biotin-labeled oligonucleotide probe, and deionized water and run at 120 V in 0.5 × Tris-borate-EDTA agarose gel for 45 minutes. The gel was transferred to a Pall Biodyne B membrane (Pall Corp, East Hills, NY) using standard electrophotography procedures. The oligonucleotide probe was immobilized using a UV cross-linker. Blocking buffer was applied to the membrane. Detection was performed using standard techniques with streptavidin–horseradish peroxidase (HRP), luminol/enhancer solution, and a peroxide solution. The membrane was then exposed to film (Hyperfilm-ECL; Amersham Biosciences, Piscataway, NJ) and developed.

**WESTERN BLOT**

Residual nuclear extracts were incubated in an equal volume of sample buffer (0.25M Tris-hydrochloride [pH 6.8], 8% Tris-glycine sodium dodecyl sulfate [SDS], 40% glycerol, 0.008% bromophenol blue, and 20% 2-mercaptoethanol), and boiled for 3 minutes. Proteins were separated using a 7.5% resolving Tris-glycine SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose by electroblotting. Membranes were then blocked in TBST (1 × Tris-buffered saline solution [TBS] and 0.1% Tween) with 5% nonfat dry milk, incubated in primary antibodies, washed 3 times for 10 minutes in TBST, incubated in secondary antibody, washed 3 times for 10 minutes in TBST, and detected using ECL Detection Reagents and HyperFilm (both from Amersham Biosciences). All antibodies were obtained from Santa Cruz Biotech (Santa Cruz, Calif). Primary antibodies were STAT3 (C-20) rabbit polyclonal IgG and p-STAT3 (Try 705) rabbit polyclonal IgG. Secondary antibody was goat antirabbit conjugated to HRP.

**QUANTIFICATION OF GEL SHIFTS**

Using Adobe Photoshop software, a histogram analysis of each STAT3 band was performed. Mean, median, and standard deviation of each band was recorded and a paired t test was performed on the data. The darker the STAT3 band was the lower the mean and median histogram score were. The standardized protein content and the probe and detection techniques used with both groups of samples through all experiments permitted valid quantitative analysis.

**RESULTS**

Gel shift assays revealed the presence of STAT3 protein in the nuclear extracts of all normal epithelium from control patients (8 of 8) and all papilloma specimens (8 of 8), but in a significantly higher concentration in the papilloma specimens (Figure 1). When mean and median histogram scores between the papilloma and normal epithelium specimens were compared, STAT3 expression in papilloma specimens was significantly stronger (P < .03). Western blot analysis confirmed that the STAT3 protein identified in the nuclear extract was activated phospho-STAT3 (Figure 2).
It is well established that HPV causes the transformation of squamous epithelium, but the exact pathways are unknown. The early region of the HPV genome is responsible for viral replication, viral transcription, and host cell transformation. Of the 8 proteins encoded in this region, E1 and E2 function to regulate viral replication and transcription; E4 may play a role in altering host cell structural proteins to assist in virion production and release; E5, E6, and E7 are transforming proteins that interact with host cell proteins and ultimately lead to unregulated cell division; and E5 has been shown to activate growth factor receptors, eg, the epidermal growth factor receptor.7

In an attempt to envisage methods by which STAT proteins can be controlled and manipulated, this study investigated their possible presence in papillomas. These proteins, which are transiently activated during normal cellular growth and differentiation,2,3 play a critical role in regulating innate and acquired host immune responses8 and their activation leads to genesis of neoplasia by promoting cellular growth, preventing apoptotic death, and modulating the host immune response.8-10

The signaling mechanisms through which HPV transforms normal epithelial cells into neoplasia is largely unknown. Recent research suggests altered STAT-protein signaling in human immunodeficiency virus–infected T lymphocytes.11 Additionally, an essential role of STAT proteins was noted in the regulation of the balance between T1 and T2 lymphocytes after immunization with viral proteins.11

Adjuvant therapies to surgical excision, eg, with indole-3-carbinol12 and intranasal cidofovir,13 are being increasingly used to slow recurrence, reduce the frequency of excision, and treat distal tracheal dissemination. The precise biological pathways of the adjuvant therapies for RRP are poorly understood, but studying the role of STAT proteins may lead to new therapies for RRP. At this time, numerous data indicate that the STAT-signaling pathway may be intricately involved with antiviral medications.14

Our results suggest that STAT3 is activated in RRP specimens at a significantly higher rate than in control tissue. This is in contrast to a previous study that suggested the level of phosphorylated STAT3 was reduced in papilloma tissue in the presence of constitutively activated epidermal growth factor receptor.15 The differences between the 2 studies may be due to a difference in methods. As a result of an extracellular signal, STAT3 proteins are activated by phosphorylation. The activated form, phospho-STAT3, has a tendency to dimerize, and only the dimerized phospho-STAT3 protein can translocate across the nuclear membrane to modulate gene expression and transcription.3 The present study assessed phospho-STAT3 protein in the nuclear extracts using an oligonucleotide probe that binds only dimerized activated phospho-STAT3 proteins in the nucleoplasm. In contrast, the previous study used a specific antibody for phospho-STAT3 protein in cell lysates, which include the cytoplasm as well as the nucleoplasm. The latter technique is less specific. It does not distinguish inactive monomers from active dimers of phospho-STAT3 protein and, more importantly, does not detect activated phospho-STAT3 protein dimers that have translocated into the nucleus to alter gene transcription.

How HPV immortalizes respiratory epithelial cells and evades host immune system detection is unclear, but modulation of the STAT-signaling pathway through the Janus kinases or through the epidermal growth factor receptor is one possible mechanism. Additional studies to investigate this possibility are ongoing in our laboratory.

STAT3 protein activation is significantly elevated in RRP lesions compared with control tissue specimens. STAT3 protein activation may be an important pathway through which HPV propagates RRP lesions. A modification of this pathway may provide novel and effective adjuvant therapies for the treatment of RRP in the future.

Submitted for publication December 4, 2003; final revision received February 25, 2004; accepted March 18, 2004.

This study was funded by an institutional grant from the Department of Otolaryngology and Communication Sciences of the Medical College of Wisconsin, Milwaukee. This study was presented in poster form at the annual meeting of the American Academy of Otolaryngology–Head and Neck Surgery; September 2003; Orlando, Fla.

Correspondence: Nalin J. Patel, MD, CHWOB, 9000 W Wisconsin Ave, Milwaukee, WI 53201 (napatel@mcw.edu).

REFERENCES