Objective: To investigate the expression of ceramide produced by sphingomyelin and DNA content in patients with healthy laryngeal mucosa, leukoplakia, and laryngeal carcinoma.

Design: A retrospective review of the clinical and surgical records of 178 patients with leukoplakia of the larynx; 23 of them developed laryngeal carcinoma.

Setting: University medical center.

Patients: One hundred seventy-eight consecutive patients with leukoplakia of the larynx were identified from the archived pathology files of the Eye Ear Nose and Throat Hospital of Fudan University from January 1, 1990, to December 30, 2001. Among them, 23 developed laryngeal carcinoma. Flow cytometry and immunohistochemistry were performed to test DNA content and ceramide expression in healthy tissue, tissue with leukoplakia, and tissue with laryngeal carcinoma from the same patient.

Results: Among 23 patients with leukoplakia, 20 had aneuploidy and 3 had diploidy. The healthy tissues were all diploids, and the tissues with laryngeal carcinoma were all aneuploids. The expression of ceramide decreased gradually from healthy tissue to tissue with leukoplakia to tissue with laryngeal carcinoma (0, no staining; 1+, weak staining; 2+, mild staining; 3+, moderate staining; 4+, strong staining; and 5+, the highest staining intensity). The expression of ceramide in DNA diploid cells is stronger than in aneuploid cells.

Conclusions: Ceramide, the second messenger in apoptosis, may associate with the progress of leukoplakia to carcinoma of the larynx. The reduction of ceramide may contribute to laryngeal carcinogenesis.

PATIENT DATA

One hundred seventy-eight consecutive patients with leukoplakia of the larynx were identified from the archival pathology files of the Eye Ear Nose and Throat Hospital of Fudan University from January 1, 1990, to December 30, 2001; 23 of these patients developed laryngeal carcinoma. We studied these 23 patients. Specimens from these patients included tissues from their healthy laryngeal mucosa, tissues with leukoplakia, and tissues with laryngeal carcinoma. The specimens were obtained using a direct laryngoscope. All of the tumors were squamous cell carcinoma. Sixteen specimens were grade 1, and 7, grade 2. Patient medical records were reviewed, and clinical data were recorded. This well-characterized panel of human tissues has been immunostained and tested by flow cytometry from the original panel tissue.

FLOW CYTOMETRIC ANALYSIS

Paraffin-stored blocks were found by flow cytometry. A suspension of isolated nuclei for the DNA ploidy analysis was prepared as described by Vindelov et al.11 The suspensions were analyzed with a flow cytometer (Epics MCL XL; Beckman Coulter, Miami, Fla), which was interfaced to a computer system multiplus (Phoenix Flow Systems, San Diego, Calif). Diploidy and aneuploidy were defined as described by Tsutsui et al.12,13 Diploidy was defined as a single G0/G1 peak on the DNA histogram. Aneuploidy was defined as a clearly distinct additional G0/G1 peak with a small G2/M peak. The DNA index of the aneuploid peak was calculated as the ratio of the DNA content of the aneuploid peak to the DNA content of the diploid peak. A multivariate analysis was performed using the Cox proportional hazards regression model. $P = .05$ was regarded as statistically significant.

IMMUNOHISTOCHEMISTRY

Well-characterized commercially available mouse monoclonal antibody to ceramide (MID 15B4; ALEXIS Biochemicals, San Diego, Calif) was used for immunohistochemistry analysis. Tissue within a block was cut in 5-µm thickness; and sections underwent deparaffinization, were washed in 0.02M phosphate-buffered saline, and were then incubated in primary antibody (dilution, 1:10) overnight in the refrigerator. After washing, sections were incubated in a second antibody (Envision; DakoCytomation Inc, Carpinteria, Calif) (dilution, 1:200) for 2 hours, then stained with $3',3'$-diaminobenzidine hydrochloride for half an hour, and observed under the light microscope. The ceramide staining was scored as the percentage of cell staining positive per high-power field. The overall intensity of the staining reaction against ceramide was scored as follows: 0, no staining; 1+, weak staining; 2+, mild staining; 3+, moderate staining; 4+, strong staining; and 5+, the highest staining intensity. For the negative control, we used phosphate-buffered saline to replace the primary antibody. The staining of the immunohistochemistry analysis was blindly assessed by 2 pathologists in the Fudan University Medical Center. Immunohistochemistry staining intensity was subjected to statistical evaluation, performed with a statistical computer program (SigmaStat; Jandel Scientific Software, San Rafael, Calif). A Kruskal-Wallis analysis of variance, a Mann-Whitney rank sum test, and a Spearman rank correlation test were performed where appropriate. $P = .05$ was considered significant.

RESULTS

HISTOGRAM OF DNA PLOIDY

Diploidy has a single G0/G1 peak on the DNA histogram (Figure 1). Aneuploidy has a clearly distinct additional G0/G1 peak with a smaller G2/M peak (Figure 2). This indicates that the aneuploid cell has
gone into the mitosis stage of the cell cycle. Cellular proliferation has begun.

**RELATIONSHIP BETWEEN DNA PLOIDY AND CARCINOGENESIS**

All 20 patients with leukoplakia who had aneuploidy developed laryngeal carcinoma; of the 158 patients with leukoplakia who had diploidy, only 3 developed laryngeal carcinoma. This indicates that the rate of carcinomatous change in aneuploid cells is higher than in diploid cells ($P<.01$).

**OVERALL STATUS OF DNA PLOIDY**

The overall status of DNA ploidy in healthy tissues, tissues with leukoplakia, and tissues with carcinoma of the larynx is as follows. All 23 healthy tissues had diploidy, while all 23 tissues with laryngeal carcinoma had aneuploidy. Finally, among 23 patients with leukoplakia whose disease progressed to laryngeal carcinoma, 20 had aneuploidy and 3 had diploidy.

**CERAMIDE STAINING INTENSITIES**

Positive labeling was observed in the cytoplasm of tested tissue (Figure 3). Strong ceramide expression was observed in healthy mucosa (mean ±SD, 4.0 ± 0.3); in contrast, weak ceramide expression was observed in tissues with laryngeal carcinoma (mean ±SD, 0.1 ± 0.1). Tissues with leukoplakia showed an intermediate level of ceramide expression (mean ±SD, 1.8 ± 0.2). A significant difference ($P<.01$) in staining intensities was found among healthy tissues (Figure 3), tissues with leukoplakia (Figure 4), and tissues with carcinoma of the larynx (Figure 5).

**COMPARISON OF STAINING INTENSITIES BETWEEN DIPLOID AND ANEUPLOID CELLS**

More intense ceramide staining was shown in diploid cells, and weaker ceramide expression was observed in aneuploid cells ($P=.01$). Therefore, the expression of ceramide in aneuploid cells is less than in diploid cells.

**COMMENT**

A model of neoplastic development has been hypothesized to involve a disruption in the balance between cell proliferation and cell death. The role of apoptosis in the regulation of tissue growth is to gain increased attention as a contributing factor in neoplastic growth. The induction of apoptosis can be initiated by such signals as cytokines, hormones, toxin exposure, and the withdrawal of various survival factors. Ceramide, produced by sphingomyelin, is the second messenger in apoptosis mediated by TNF–TNF receptor and Fas–Fas ligand. Ceramide is a key factor in this process. The decrease of ceramide, such as the glycosylation of ceramide, has been hypothesized to result in excessive cellular accumulation, leading to various pathological processes, such as inflammation proliferation and precancerous lesions. To understand the function of ceramide from precancerous lesions to carcinoma of the larynx, we investigated the expression of ceramide. In our retrospective study, ceramide expression gradually decreased from healthy laryngeal mucosa to tissues with leukoplakia to tissues with carcinoma of the larynx. A significant difference in ceramide expression has been found between each of them ($P<.05$). Because of the decrease of ceramide expression, the process of apoptosis is blocked. The balance of proliferation and apoptosis breaks. Therefore, healthy mucosa progressed into precancerous lesions and leukoplakia progressed into laryngeal cancer. Similar results were reported in a study of breast cancer and colon carcinoma.

DNA ploidy has been analyzed as a prognostic, or predictive, factor for head and neck squamous cell carcinoma. The most important significance of DNA ploidy is as a biological variable that indicates the aggressiveness of cancer cells. We found that the incidence of
carcinomatous change in aneuploid cells is much higher than in diploid cells (P<.01). DNA aneuploidy may be a marker in the early stage of carcinomatous change. The expression of ceramide in aneuploid cells is less than in diploid cells. The staining intensity was as follows: diploid cells in healthy tissues (n = 20), 4.0 ± 0.2; diploid cells in tissues with leukoplakia (n = 3), 3.1 ± 0.2; and aneuploid cells in tissues with leukoplakia (n = 20), 1.5 ± 0.1. Reduction of ceramide expression in patients with laryngeal carcinoma resulted in the blockage of apoptosis.

Several studies have shown that sphingolipids control the balance in cells between growth and proliferation and cell death by apoptosis. Sphingosine-1-phosphate and glucosylceramide induce the proliferation process; and ceramide, a metabolic intermediate between the 2, induces cell death by apoptosis.16 In cancers, the balance seems to have come undone and it should be possible to kill the cells by enhancing the processes that lead to ceramide accumulation. The 2 control systems are intertwined, modulated by many different agents affecting the activities of the enzymes in ceramide–glucosylceramide–sphingosine-1-phosphate interdependence.10 It has been proposed that successful cancer chemotherapy requires the use of many agents to elevate ceramide levels adequately. Inherent or acquired drug resistance, which frequently characterizes cancer cells, is caused by multiple mechanisms, including dysfunctional metabolism of the lipid second messenger, ceramide.17 Ceramide, the basic structural unit of the sphingolipids, plays a role in activating cell death signals initiated by cytokines, chemotherapeutic agents, and ionizing radiation.17

Recent research about the metabolism of ceramide suggests that this agent may have an important influence on the effectiveness of various cancer therapeutic agents. In particular, the cytotoxic effect of chemotherapy is decreased when the generation of ceramide is impaired, but is increased when the degradation of ceramide is blocked.17 It has been proposed that successful cancer chemotherapy requires the use of many agents to elevate the ceramide level adequately.

In conclusion, ceramide, the second messenger in apoptosis, may associate with the progress of precancerous lesions to laryngeal cancer. Elevating the intracellular content of ceramide will be a new way to interfere with carcinogenesis.

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