INTRODUCTION

Apoptosis is an important mechanism in the maintenance of a healthy immune system and is often dysregulated in neoplastic transformation. Cell-to-cell interactions between T cells and other cells of the immune system, as well as cytokines, play an important role in the apoptosis of B cells. When this mechanism is suppressed, it can result in tumorigenesis.

Tumor suppressor genes such as DAPK, p16, p73, Rb, and others function by promoting apoptosis or suppressing growth and are often deleted, mutated, or hypermethylated in tumor cells. Hypermethylation of the promoter region of a gene inactivates it at the level of transcription (1). Promoter region hypermethylation has been used as a method for molecular detection of tumor cells in a background of normal cells (1–8).

DAPK is a 160-kDa calcium/calmodulin-dependent serine threonine kinase protein that has been shown to play a role in the apoptosis pathways downstream to IFN-γ, TNF-α, transforming growth factor-β, and Fas (9–13). DAPK was initially identified as a mediator of cell death in the apoptosis pathway induced by IFN-γ and mapped to chromosome 9q34. It has a death domain at its COOH-terminal end that is critical to its function (12). Hypermethylation of DAPK has been shown to be an important epigenetic alteration in lung, esophageal, head and neck, prostate, bladder, and gastric malignancies (12–18). It has been shown to occur in several B-cell malignancies as well, including B-cell lymphoma, multiple myeloma, and Burkitt’s lymphoma (13, 19–21).

Although evasion of apoptosis has gained a great deal of attention as a mechanism of tumorigenesis, it plays an equally important physiological role in preventing death of normal cells. During a community-based upper aerodigestive tract cancer screening study, we noted that a significant proportion of subjects, without evidence of any malignancy, had low but detectable levels of DAPK promoter hypermethylation in their peripheral blood cells. Using Q-MSP, we investigated whether there is a specific subpopulation of these peripheral blood cells that have promoter region hypermethylation of the DAPK gene in normal individuals. We were able to demonstrate localization of DAPK promoter hypermethylation to a specific compartment of the B-cell population. This may imply that DAPK promoter hypermethylation plays a physiological role in a subpopulation of normal B cells and that B-cell malignancies with DAPK hypermethylation may arise as a clonal outgrowth from this subpopulation. In addition, DAPK hypermethylated lymphocytes may act as a confounding factor in tumor detection methods that use DAPK hypermethylation as a tumor marker.

MATERIALS AND METHODS

Sample Collection. The experimental protocol was approved by the Johns Hopkins Medical Institutions Institutional Review Board. Local community organizations with a significant proportion of members with tobacco exposure were contacted for participation in a screening study of upper aerodigestive tract malignancies. According to institutional review board approved protocol, informed consent was obtained from all enrolled subjects. A total of 143 healthy patients of a range of ages and ethnic backgrounds were enrolled. At the initial screening, all subjects were administered a confidential survey of risk factors for upper aerodigestive tract malignancies, including alcohol and tobacco use, as well as the presence of comorbid illnesses. Smoking was defined as use of tobacco, chewable or smokable, for at least 1 year continuously. Heavy alcohol use was defined as intake of more than two alcoholic drinks per day. A thorough head and neck examination, including cranial nerve function; palpation of cervical, thyroid, and parotid nodal basins; visual inspection and palpation of the oral cavity and oropharynx; and indirect mirror laryngoscopy or flexible fiberoptic laryngoscopy were performed. In addition, a sample of blood was collected and separated into serum and cellular components for later analysis. Analysis of collected samples included assays for DAPK promoter hypermethylation in peripheral blood cell DNA.

FACS. Normal peripheral blood cells were obtained from whole blood for analysis. In the samples of peripheral blood used to sort subpopulations of leukocytes, a double Ficoll (Ficoll-Hypaque 1077 and 1119; Sigma) procedure was performed to isolate mononuclear and granulocytic cells. The cells were washed with RPMI, supplemented with 10% fetal bovine serum, counted with
a hemacytometer, and resuspended in a mixture of PBS, BSA, and azide (0.01%) at a concentration of 2 × 10^6 cells/ml.

Fluorescently labeled antibodies were added to each sample according to the respective manufacturer’s recommended protocols. Cells were incubated for 30–60 min at 4°C in the dark. Samples were then washed with 2 ml of PBS-BSA-azide, resuspended in 0.5 ml of 10% FBS in RPMI, and kept on ice until sorting.

For each experiment, all control conditions were analyzed, including no stain, isotype control, and single fluorescence. All of the antibodies used were manufactured by Becton-Dickinson (analytical grade or BD PharMingen research grade).

Leukocyte subpopulations were sorted using fluorescently labeled antibodies CD45-FITC, CD33-PE, CD3/CD19 reagent, CD27-PE, IgM-FITC, CD19-PE, and IgG-FITC, with measurement by SSC, FSC, and FL-1. The granulocytes were sorted based on CD45-FITC and SSC. The monocytes were sorted by use of CD45-FITC, SSC, and CD33-PE. The T- and B-cell populations were sorted by use of the CD3/CD19 reagent. IgM+ B cells were sorted by use of IgM-FITC, CD19-PE, SSC, and FSC; IgG+ B cells were sorted by use of IgG-FITC, CD19-PE, SSC, and FSC.

**DNA Extraction.** Peripheral blood cells and FACS leukocyte subpopulations were centrifuged at 2500 rpm for 15 min, and the cell pellets were digested with 1% proteinase K in 10% SDS for 72 h at 48°C. DNA was then extracted with phenol–chloroform and precipitated with alcohol.

**Bisulfite Treatment.** Sodium bisulfite treatment was carried out according to a previously published protocol (7). Samples were incubated in sodium bisulfite at 70°C for 3 h before purification with use of a Wizard purifcation resin (Promega, Madison, WI). Bisulfite-treated DNA was resuspended in 40 μl of 5 mM Tris–HCl buffer (pH 8.0). Normal leukocyte DNA from a donor was methylated using Sss1 methyltransferase (New England Biolabs Inc., Beverly, MA) to generate DNA that was methylated at all CpG dinucleotides and was used as hypermethylated (positive) control.

**Primers and Probes.** Forward and reverse primers and fluorescently labeled hybridization probes for both hypermethylated DAPK promoter and β-actin published in previous studies were used for the Q-MSP assay (7). The primers and probe for β-actin were designed to amplify a region without CpG dinucleotides so that PCR amplification would occur irrespective of methylation status. The forward primer for the methylation MSP assay, 5′-ggtatgggtggtgaatatgccgt-3′, was the same as that for Q-MSP, and the reverse primer, 5′-agtttttaggcgttcgggt-3′, was designed using Primer3 software. The MSP primers encompassed a 226-bp segment of the DAPK promoter CpG island with 26 CpG dinucleotides. Primers were obtained from Applied Biosystems (Carlsbad, CA).

PCR reactions were carried out in a 384-well plate in a 20-μl final volume/well, containing: 600 nM each of the forward and reverse primers; 16.6 mM ammonium sulfate; 67 mM Tris; 2.5 mM MgCl2; 10 mM β-mercaptoethanol; and 0.1% DMSO. We used 5 μl of bisulfite-treated DNA solution for each reaction. Methylated DNA control solutions and distilled water were used as positive and negative controls, respectively, with each set of reactions. MSP reaction products were run on a standard 1.5% agarose gel along with a 1-kbp+ ladder. Bands at >226 bp were excised, and DNA was extracted using a QIAquick gel extraction kit from Qiagen (Valencia, CA). The extracted DNA was sent to the Biosynthesis & Sequencing facility at Johns Hopkins University School of Medicine for sequencing.

**Data Analysis.** For each DNA sample, the mean value of five replicate hypermethylated DAPK PCR reactions and duplicate β-actin PCR reactions were used for the analysis. Occasional single outliers for DAPK, representing failed PCR, were excluded, and the remaining replicates were used for the analysis. Because there are two copies of DAPK and β-actin per genome, the percentage of cells with DAPK hypermethylation was calculated by dividing the mean amount of methylated DAPK promoter into the mean total DNA content of each sample, represented by the amount of β-actin, a housekeeping gene. Data were analyzed using Microsoft Excel software.

Statistical analysis was carried out using Student’s t test to determine whether there was any association between peripheral blood cell DAPK promoter hypermethylation and age, gender, detection of abnormality in the physical examination, smoking status, and/or ethanol intake.

**RESULTS**

**DAPK Promoter Hypermethylation in Peripheral Blood Cells of Normal Subjects.** A total of 143 healthy individuals, as outlined in the “Materials and Methods” above, were enrolled in the study. See Table 1 for the demographic breakdown of the subject population. Of the 143 subjects analyzed by Q-MSP, 75 (52%) showed low levels of DAPK promoter hypermethylation in their peripheral blood cells, ranging from 0.003% to 1.181%. Peripheral blood cells from 10 randomly selected patients with DAPK promoter hypermethylation values in the range of 0.253–1.181% were further analyzed by MSP and sequencing. A 226-bp region at the beginning portion of the promoter region CpG island, containing 26 CpG dinucleotides, was amplified. Sequencing demonstrated dense hypermethylation of the amplified segment (see Figs. 1 and 2). In all patients, >80% of the CpG dinucleotides were methylated. There was no statistically significant correlation between peripheral blood cell DAPK promoter hypermethylation and age, gender, detection of abnormality in the physical examination, smoking status, and/or ethanol intake.

Peripheral blood cells were collected from three subjects from the
DAPK methylation specific PCR product

Fig. 1. Representative DNA sequence of the 226-bp MSP product from the DAPK promoter CpG island. The non-bisulfite-treated sequence is 5′-ccgccccgccccgctagtctc-3′. In human DNA, only cytosines that are 5′ to guanosines, i.e., that are part of CpG dinucleotides, are methylated. Bisulfite treatment and MSP converts unmethylated cytosines to thymines. None of the CpG dinucleotides were converted to TpG dinucleotides.

DAPK promoter hypermethylation could play in tumor detection (1–8).

DAPK promoter hypermethylation may play a physiological role in B cells as well. Cell proliferation and apoptosis are essential to the normal function of the immune system. B-cell populations proliferate in response to antigenic stimulation and recede at the end of an immune response. If one or both of the above mechanisms malfunction, autoimmunity, immunodeficiency, or malignancy can ensue (23, 24). Normal functioning of proapoptotic proteins such as Fas, Fas ligand, and DAPK are essential for physiological apoptotic control of B-cell populations (23–25). DAPK promoter hypermethylation occurs in many solid B-cell malignancies, including B-cell lymphomas (13, 19–21). Here we show that DAPK promoter hypermethylation occurs in a subpopulation of healthy B cells as well and may play an as yet unknown physiological role. The presence of methylated tumor suppressor gene promoters in normal lymphocytes has been documented for p15INK4B in a smaller sample size (n = 10), and the description of this phenomenon in a larger population for a different gene promoter raises the possibility that this may be a common phenomenon in peripheral lymphocytes from normal individuals (26).

In 143 normal subjects, we found that 52% (75 of 143) had low-grade DAPK promoter hypermethylation detectable in their peripheral blood cells, ranging from 0.003 to 1.181%. Several investigations have been carried out regarding the utility of using hypermethylated DAPK promoter as a tumor marker in tumor detection (1–8). In some previous studies, peripheral blood cells from normal patients were used as negative controls or were found to be negative for DAPK hypermethylation (19, 21). These studies used MSP, not Q-MSP. In addition, the low-level DAPK hypermethylation picked up in peripheral blood cells in this study was detected occasionally beyond PCR cycle number 40. In addition, we detected DAPK promoter hypermethylation with both MSP and Q-MSP and confirmed dense hypermethylation by sequencing the PCR product.

In our analysis of the various subpopulations of leukocytes (Figs. 3 and 4), it is clear that DAPK hypermethylation is predominantly present in the B-cell population. Although signal is present in the other leukocyte subpopulations, DAPK hypermethylation is significantly less relative to the B-cell population. This may indicate that a small fraction of peripheral blood cells outside of the B-cell compartment also have undergone DAPK promoter hypermethylation or that this is simply an artifact produced by incomplete sorting of the B-cell compartment by FACS (Figs. 3 and 4). Our results also show that a significantly higher proportion of peripheral IgM− B cells have DAPK hypermethylation than peripheral IgM+ B cells (Fig. 5) and that a slightly higher proportion of peripheral IgG+ B cells have DAPK promoter hypermethylation.
DAPK hypermethylation than peripheral IgG− B cells in one individual.

The presence of DAPK promoter hypermethylation in IgM− B cells from normal individuals may indicate that B-cell malignancies with the same hypermethylation arise from a subpopulation of these cells. Identification of these specific cells could have implications in tumor detection as well as in tumor therapy. Lymphocytes are present in all tissues of the human body, except in the central nervous system. With a fraction of them normally being hypermethylated at the DAPK locus, as we have shown, they have the potential to act as confounding factors in tumor detection techniques that use DAPK hypermethylation as a tumor marker.

At this time we can only speculate on the role that DAPK hypermethylation plays in the normal physiology of B-cell development. DAPK acts downstream to caspase 8 in the apoptotic pathways of Fas, TNF-α, and IFN-γ (9–12, 14, 15). Fas and TNF-α are important mediators of B-cell death (13). In the signal-rich environments of bone marrow and lymph nodes, signals for apoptosis and cell survival occur in a milieu. Depending on a combination of signals, a given B cell will proliferate or die. For example, if the membrane receptor in a germinal center B cell is activated by Fas-L from a T cell or from a follicular dendritic cell, it will undergo apoptosis. However, if another membrane receptor, CD40, is activated concurrently, the B cell evades apoptosis (23–25). It is not implausible to conjecture that hypermethylation of DAPK in certain subpopulations of B cells may serve as a rescue signal from apoptosis mediated by Fas, TNF-α, and/or IFN-γ.

Fig. 3. Q-MSP amplification curves. log Δfluorescence versus PCR cycle, for β-actin and hypermethylated DAPK promoter in peripheral blood cells (A), peripheral mononuclear cells (B), T cells (C), B cells (D), and monocytes (E) from subject B. Two representative curves from each sample are depicted. Also shown are the standard curves for DAPK and β-actin (F). Note that the earlier the cycle at which an amplification curve crosses the threshold (C_{T}), the greater the amount of template present initially in the PCR reaction. The C_{T} for B-cell β-actin is higher than all others except monocytes, indicating less total DNA content. Only peripheral blood cells, B cells, and T cells showed amplification of hypermethylated DAPK. The C_{T} for B cells with hypermethylated DAPK is comparable to or lower than that of peripheral blood cells and T cells, although much larger quantities of DNA were used in the latter reactions, indicating a higher proportion of B cells with hypermethylated DAPK.

Fig. 4. Percentage of DAPK promoter hypermethylation versus subpopulation of leukocytes. In subjects 9, 10, and 17, the value for B cells ranges from 1.074 to 6.026%, whereas for other subpopulations, it is <0.06%. Monos, monocytes; PMNs, polymorphonuclear cells.

Fig. 5. Percentage of DAPK promoter hypermethylation in IgM+ and IgM− B cells. The percentage of DAPK promoter hypermethylation in each subpopulation is depicted for subject 10.

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Death-Associated Protein Kinase Promoter Hypermethylation in Normal Human Lymphocytes

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