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Graft-versus-Host Disease: Role of Inflammation in the Development of Chromosomal Abnormalities of Keratinocytes

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Graft-versus-host disease (GVHD) is a major risk factor for secondary malignancy after hematopoietic stem cell transplantation. Squamous cell carcinoma (SCC) of the skin and mucous membranes are especially frequent in this setting where aneuploidy and tetraploidy are associated with aggressive disease. The current study is directed at the mechanism of neoplasia in this setting. Unmanipulated keratinocytes from areas of oral GVHD in 9 patients showed tetraploidy in 10% to 46% of cells when examined by florescein in situ hybridization (FISH). Keratinocytes isolated from biopsy sites of GVHD but not from normal tissue showed even greater numbers of tetraploid cells (mean = 78%, range: 15%-85%; N = 9) after culture. To mimic the inflammatory process in GVHD, allogeneic HLA-mismatched lymphocytes were mixed with normal keratinocytes. After 2 weeks, substantial numbers of aneuploid and tetraploid cells were evident in cultures with lymphocytes and with purified CD8 but not CD4 cells. Telomere length was substantially decreased in the lymphocyte-treated sample. No mutations were present in the p53 gene, although haploinsufficiency for p53 due to the loss of chromosome 17 was common in cells exposed to lymphocytes. These findings suggest that in GVHD, inflammation and repeated cell division correlate with the development of karyotypic abnormalities.

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INTRODUCTION

Recipients of solid organ and bone marrow transplants (BMTs) are at risk of developing solid tumors. Two percent to 6% of long-term survivors acquire some type of malignancy by 10 years of follow-up [1-4]. Although radiation therapy increases the risk of nonsquamous cell carcinomas of breast, brain, and bone, and of melanoma [5,6], chronic graft-versus-host disease (cGVHD) and its therapy increase the probability of developing squamous cell carcinoma (SCC) of the skin and oral cavity [7]. In one study, the risk for SCC among transplant recipients with cGVHD was 3-fold that of patients without GVHD [7]. Major risk factors included a long duration of cGVHD therapy, use of azathioprine, and severity of cGVHD; the conditioning regimen or the use of high-dose radiation did not influence the incidence of SCC. In BMT patients, the relative contributions of immunosuppression and the inflammatory effects of cGVHD cannot be ascertained. However, in patients receiving solid organ transplants, immunosuppression alone has been associated with SCC, and a randomized trial of immunosuppression reduction showed significantly fewer cancers in those receiving the less intense regimen [8,9].

Chronic inflammation alone is associated with increased risk of cancer in some conditions. Examples include bowel cancer in ulcerative colitis [10], esophageal cancer in Barrett's esophagitis [11], and hepatoma in hepatitis B [12]. DNA damage in areas of inflammation is believed to be related to reactive oxygen species [13-15], and in vitro exposure to endogenous oxidants

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leads to degradation of deoxyribose residues [13,16]. Polypoid and aneuploid cells are evident at sites of inflammation preceding the development of cancer in Barrett's esophagus [17], in hepatocytes after partial hepatectomy [18], in ulcerative colitis [10], and transiently in skin wounds and burns [19]. Mutation or loss of a pivotal oncogene, *TP53* [20], also occurs in areas of inflammation [21]. *TP53* is a checkpoint gene that forces cells with substantial DNA damage into senescence or apoptosis [22,23]. Approximately 85% of colon cancers resulting from inflammatory bowel disease have lost at least 1 *TP53* allele [21]. Haploinsufficiency, gene duplication, or mutation of *TP53* allows cells with polyploidy to continue to live and divide [24]. It has been theorized that in inflammatory disorders, repeated oxidative stress results in *TP53* mutation or loss [21]. An alternative mechanism for the development of ploidy involves telomeric shortening resulting from repeated cell division accompanying tissue repair [25]. In the telomerase-deficient mouse, significant telomeric shortening results in end-to-end fusion of chromatids with consequent nondisjunction during metaphase and aneuploidy [26].

Tetraploidy and aneuploidy are present in most cases of SCC and complex chromosomal abnormalities correlate with poor prognosis [27-29]. Tetraploidy results from chromosome nondisjunction in late mitosis SCC [28,30-34] and precedes the development of aneuploidy in some models [26]. In this study, we examined sites of oral and skin GVHD in transplant patients and developed an in vitro model of GVHD using keratinocytes cocultured with HLA mismatched allogeneic lymphocytes or inflammatory cytokines. We also assessed the effect of inflammation on telomere length and *TP53* in an effort to develop a mechanism for the occurrence of SCC in patients with GVHD.

MATERIALS AND METHODS

Patients

Patients undergoing hematopoietic stem cell transplantation (HSCT) were recruited for the study using a protocol approved by the institutional review board of the National Heart, Lung, and Blood Institute, after written informed consent. All patients were adults with a hematologic disorders who had received a granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood allograft from a fully HLA-matched male sibling donor. Some patients received full myeloablation with total body irradiation (TBI) (1360 rad) and cyclophosphamide (Cy) 60 mg/kg/day for 2 days, followed by infusion of grafts that were T cell depleted by positive selection for $CD34^+$ cells via the Isolex immunoabsorption device. Other patients received nonmanipulated G-CSF mobilized peripheral blood cells following a nonmyeloablative conditioning regimen of fludarabine and Cy. Samples were obtained from patients who were transplanted a minimum of 2 months before the biopsy and were confirmed to have full donor engraftment by BM cytogenetics or by polymerase chain reaction (PCR)-based microsatellite chimerism analysis of the peripheral blood. Patients were required to have either active biopsy-proven cutaneous GVHD at the time of skin biopsy or oral buccal mucosal smear. Patients with oral GVHD were diagnosed on clinical grounds.

Cell Preparation and Tissue Culture

Buccal mucosal cells

Buccal mucosal cells were obtained by scraping the area of the oral mucosa that appeared to be most involved with GVHD. Cells were placed directly on the slide, after which they were fixed and prepared for FISH. Generally, several mucosal scrapings were obtained from the patient so that many cells could be examined. Buccal mucosal cells were also obtained from 10 healthy volunteers.

Skin biopsies from GVHD patients

Two contiguous 4 to 6 mm punch biopsy samples from a site of ongoing or previous cutaneous GVHD were obtained from each patient with significant GVHD by standard techniques [35]. One specimen was fixed in formalin and embedded in paraffin for routine histologic evaluation. The second specimen was treated at 4°C overnight with dispase (Becton-Dickinson Labware, Bedford, MA, USA), a type IV collagenase, to separate the epidermal layer from the basement membrane. The epidermal sheet was then separated mechanically from the dermal layer and cells were dissociated by shaking and digesting with 0.5% trypsin-0.53 mM EDTA (Gibco BRL, Gaithersburg, MD, USA) at 37°C. The cells were plated on tissue culture dishes in keratinocytes-serum-free media (Gibco BRL) supplemented with bovine pituitary extract and recombinant epidermal growth factor. Cells were expanded to larger flasks using trypsinization when the monolayers reached 60% to 70% confluence.

Culture of keratinocytes obtained from foreskins

To study normal keratinocytes, we obtained thoses isolated from foreskin fibroblasts from Carole Yee et al. (isolation detailed elsewhere) [35] and cultured as previously described in keratinocyte growth media [35]. To assess the effect of inflammation, one flask of confluent keratinocytes was cocultured with normal peripheral blood mononuclear cells with or without interferon-gamma (IFN-γ) (1000 units/mL), or in some cases with isolated CD4 or CD8 cells. Nonadherent lymphocytes were removed after 48 hours at 37°C, and fresh media was added to the keratinocytes. Keratinocytes