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 fluorescein 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 non-squamous 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
leads to degradation of deoxyribose residues [13,16]. Polyploid 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 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
were allowed to become 70% to 80% confluent and passaged two times before preparation for fluorescence in situ hybridization (FISH).

**FISH**

Keratinocytes were exposed to trypsin, enumerated, and fixed to glass slides. Cells were incubated in prewarmed hypotonic solution (0.3% KCl sol) at 37°C for 30 minutes, spun, and resuspended in Carnoy’s fixative (3 parts methanol and 1 part glacial acetic acid) three times. Fixed cells were dropped onto cleaned glass slides and dried overnight. Before hybridization, slides were incubated in 2×SSC for 2 minutes at 73°C, then digested with pepsin for 5 minutes at 37°C and dehydrated in serial ethanol washes. CEP combined X/Y (spectrum orange and spectrum green, respectively) centromeric probe (Vysis, Inc, Downers Grove, IL, USA) was used for markers of tetraploidy because it is capable of distinguishing fusion cells from cells that are tetraploid secondary to failure of cytokinesis. Three hundred cells were scored by three “blinded” observers on all samples except for the buccal smears, in which all available cells were counted. Cells were scored according to current guidelines [36]; cells containing twice the normal complement of stained chromosomes were defined as tetraploid; other abnormal gains or losses were defined as aneuploid.

Centromeric probes for chromosomes 7, 8, and 9 (Abbott Laboratories, Abbott Park, IL, USA) were used for experiments with normal keratinocytes. Locus-specific p53 probe (Abbott) was used to assess loss of heterozygosity in keratinocytes. Slides were placed on the Hybrite hybridization system (Abbott), and the samples were denatured at 73°C for 2 minutes. After overnight hybridization at 42°C, the slides were washed in prewarmed 0.4×SSC at 73°C for 2 minutes and 2×SSC/0.1% NP-40 at room temperature for 1 minute. The slides were dried in the dark, and then counterstained with DAPI-I before examination using a fluorescence microscope. The overnight hybridization temperature for the TP53 locus specific probe was 37°C.

**DNA Cell Cycle Analysis**

Treated and untreated cells were prepared as directed by the NuCycle Dapi Kit (Exalpha Biologicals, Maynard, MA, USA) and analyzed on a Beckman Coulter FC 500 cytometer.

**Cytokine Analysis**

Supernatants from keratinocytes cocultured in the presence of CD8+ T cells, CD4+ T cells, or in the absence of T cells (negative control) were analyzed for the following cytokines: chemokine ligand 5 (CXCL5), CXCL11, thrombopoietin (Tpo), tumor necrosis factor-alpha (TNF-α), G-CSF, epidermal growth factor (EGF), hepatocyte growth factor (HGF), interferon-inducible cytokine-10 (IP-10), interleukin 1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-6, IL-8, vascular endothelial growth factor (VEGF), eotaxin, CD40 ligand (CD40L), leptin, chemokine ligand 5 (RANTES), macrophage inflammatory protein-1 alpha (MIP-1α), and MIP-1ß. Measurement of all cytokines was performed simultaneously by an immunobead-based multiplex assay (Luminex) according to the manufacturer’s instructions. Panels of capture antibody-coated beads and labeled detection antibodies were acquired from R&D Systems, Inc. (Minneapolis, MN, USA).

**qPCR for Measurement of Telomere Length**

Telomere length of pretreatment peripheral blood leukocytes was assessed by quantitative polymerase chain reaction (qPCR) as previously described [12,13]. Total leukocytes were separated by ammonium-based lysis of red blood cells and DNA was extracted using the DNeasy Blood kit (Qiagen, Chatsworth, CA, USA). PCRs were performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each sample’s telomere length (x) was based on the telomere to single copy gene ratio (T/S ratio) and on the calculation of the ΔCt, [C(telomeres)/C(single gene)]. Telomere length was expressed as relative T/S ratio, which was normalized to the average T/S ratio of the reference sample [2−(ΔCttx−ΔCttx) = 2−ΔΔCt].

**P53 Sequencing of Keratinocytes**

Screening for TP53 mutations in patients was carried out using direct sequencing in both directions. PCR primers were designed to amplify exons 5, 6, 7, 8, and 9 of the TP53 gene (ENSG00000141510) from genomic DNA as 3 separate amplicons. The primer sequences are available on request. The PCR products were analyzed using 3730xl DNA analyzer (Applied Biosystems).

**RESULTS**

Unmanipulated, uncultured buccal mucosal cells from sites of GVHD show significant numbers of tetraploid cells.

To determine if chromosomal abnormalities could be detected in areas of the oral mucosa affected by GVHD, we obtained buccal mucosal cells from patients with and without GVHD and performed FISH using centromeric probes for X/Y. Cells were placed directly on a slide, fixed, and then stained with a FISH X/Y probe. The X/Y probe was chosen for our transplant patient experiments to distinguish tetraploid cells from fusion cells (in patients with sex-discordant transplants (XY/XY or XX/XX) from fusion cells (XY/XX)). When FISH was performed...
on multiple unmanipulated buccal smears obtained from 9 patients at sites of oral GVHD, all demonstrated small numbers of tetraploid cells. None of the 6 BMT patients without a recent history of GVHD or the 10 healthy controls (ranging in age from 20 to 67 years) showed tetraploidy (Table 1). None of the 4 patients from the GVHD cohort who had sex-discordant transplants demonstrated XX/XY fusion cells. Punch biopsies were obtained from individuals with biopsy-proven GVHD, fixed, embedded in paraffin, and prepared for FISH as previously described [35]. It has been shown that tissue repair and active mitosis occur in areas recovering from GVHD. To determine if repeated cell divisions predispose toward tetraploidy or aneuploidy, we studied keratinocytes isolated from biopsies of patients at sites of GVHD and from normal foreskins. Keratinocytes obtained from areas of GVHD in patients with sex-discordant transplants were cultured in epithelial growth factor media for 2 weeks (the media does not support growth of hematopoietic cells or fibroblasts), passed 2 times in 75 cm² flasks, and subjected to FISH. Tetraploidy was observed in many of the cultures (N = 9; mean = 45%) (Table 2). There was no evidence of fusion cells in the 4 patients who received sex-discordant transplants stained with X/Y probe (Table 2; patients 6, 7, 8, and 9). When skin biopsies obtained from patients with GVHD and the healthy keratinocytes were expanded, tetraploidy was even more pronounced in patient samples (Table 2). Simple expansion of keratinocytes derived from normal foreskins did not result in significant tetraploidy or aneuploidy.

Table 1. Karyotype Analysis of Buccal Mucosal Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>GVHD</th>
<th>Donor Sex Discordant</th>
<th>Time from Transplant</th>
<th>Transplant Regimen</th>
<th>% Tetraploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Chronic systemic</td>
<td>No</td>
<td>18 months</td>
<td>Flu/Cy</td>
<td>23/50 (46%)</td>
</tr>
<tr>
<td>12</td>
<td>Acute skin IV</td>
<td>Yes</td>
<td>5 months</td>
<td>Flu/Cy</td>
<td>5/25 (20%)</td>
</tr>
<tr>
<td>13</td>
<td>Chronic limited</td>
<td>No</td>
<td>20 months</td>
<td>Flu/Cy</td>
<td>5/20 (25%)</td>
</tr>
<tr>
<td>14</td>
<td>Chronic limited</td>
<td>Yes</td>
<td>38 months</td>
<td>Flu/Cy</td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td>15</td>
<td>Acute skin I</td>
<td>Yes</td>
<td>2.5 months</td>
<td>Flu/Cy</td>
<td>2/20 (10%)</td>
</tr>
<tr>
<td>16</td>
<td>Acute skin I</td>
<td>Yes</td>
<td>4 months</td>
<td>Flu/Cy</td>
<td>3/7 (8%)</td>
</tr>
<tr>
<td>17</td>
<td>Chronic limited</td>
<td>No</td>
<td>4 months</td>
<td>Flu/Cy</td>
<td>6/30 (20%)</td>
</tr>
<tr>
<td>18</td>
<td>Chronic limited</td>
<td>No</td>
<td>1.3 months</td>
<td>Flu/Cy</td>
<td>3/25 (12%)</td>
</tr>
<tr>
<td>19</td>
<td>None</td>
<td>No</td>
<td>6 months</td>
<td>Flu/Cy</td>
<td>0/72 (0%)</td>
</tr>
<tr>
<td>20</td>
<td>None</td>
<td>No</td>
<td>2 months</td>
<td>Flu/Cy</td>
<td>0/21 (0%)</td>
</tr>
<tr>
<td>21</td>
<td>None</td>
<td>No</td>
<td>22 months</td>
<td>Flu/Cy</td>
<td>0/3 (0%)</td>
</tr>
<tr>
<td>22</td>
<td>None</td>
<td>No</td>
<td>2.6 months</td>
<td>Flu/Cy</td>
<td>0/37 (0%)</td>
</tr>
<tr>
<td>23</td>
<td>None</td>
<td>No</td>
<td>36 months</td>
<td>Flu/Cy</td>
<td>0/11 (0%)</td>
</tr>
<tr>
<td>24</td>
<td>None</td>
<td>No</td>
<td>12 months</td>
<td>Flu/Cy</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td>25</td>
<td>None</td>
<td>No</td>
<td>—</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>N = 10</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0</td>
</tr>
</tbody>
</table>

GVHD indicates graft-versus-host disease; Flu/Cy, fludarabine/cyclophosphamide.
Buccal mucosal smears were obtained from 8 patients from areas of GVHD and 6 patients from uninvolved areas of the buccal mucosa. Keratinocytes from GVHD sites forced into repeated cell division by multiple in vitro passaging show even more pronounced tetraploidy than unmanipulated keratinocytes.

Table 2. Karyotype Analysis of Biopsies of Skin in GVHD Patient Skin Keratinocytes Following Expansion

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Dx</th>
<th>Conditioning</th>
<th>Years after Transplant</th>
<th>GVHD</th>
<th>GVHD on bx</th>
<th>Before Expansion</th>
<th>Following Expansion</th>
<th>% Tetraploidy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>AML</td>
<td>TBI/Cy</td>
<td>3</td>
<td>I</td>
<td>No</td>
<td>1</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>CML</td>
<td>TBI/Cy</td>
<td>1</td>
<td>II</td>
<td>No</td>
<td>5</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>AML</td>
<td>TBI/Cy</td>
<td>2</td>
<td>II</td>
<td>Yes</td>
<td>12</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>CLL</td>
<td>TBI/Cy</td>
<td>3</td>
<td>IV</td>
<td>Yes</td>
<td>3</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>NHL</td>
<td>Flui/Cy</td>
<td>1</td>
<td>I</td>
<td>No</td>
<td>4</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>CML</td>
<td>Flui/Cy</td>
<td>2</td>
<td>III</td>
<td>No</td>
<td>13</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>47</td>
<td>MDS</td>
<td>TBI/Cy</td>
<td>7</td>
<td>I</td>
<td>No</td>
<td>2</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>PNH</td>
<td>Flui/Cy</td>
<td>0.6</td>
<td>II</td>
<td>Yes</td>
<td>5</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>CML</td>
<td>TBI/Cy</td>
<td>6</td>
<td>II</td>
<td>No</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>N = 20</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>1-3</td>
<td></td>
</tr>
</tbody>
</table>

GVHD indicates graft-versus-host disease; Fludarabine/cyclophosphamide; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's leukemia; CLL, chronic lymphocytic leukemia; AML, acute myelogenous leukemia; PNH, paroxysmal nocturnal hemoglobinuria; TBI/Cy, total body irradiation and cyclophosphamide.

Keratinocytes obtained from skin biopsies of patients with a history of cutaneous GVHD were expanded through two passages in 75 cm² flasks and FISH performed using probes for chromosomes X and Y. The last column represents the tetraploidy observed prior to and following expansion of cells. Percent tetraploidy prior to expansion is based on counts done on paraffin-sections stained with X/Y probe. Tetraploidy in expanded keratinocytes obtained from normals ranged from 0% to 3%, with a median of 2% following scoring of 300 cells.

*Percentages were based on cell counts of 300.
Keratinocytes Develop Tetraploidy and Aneuploidy after Coculture with Peripheral Blood Mononuclear Cells (PBMCs)

To develop a model for GVHD in which individual and potentially inciting factors could be controlled, we examined the effect of HLA-mismatched allogeneic lymphocytes on the development of ploidy in keratinocytes in vitro. Keratinocytes derived from foreskins, were cultured with allogeneic lymphocytes for 2 days, after which the nonadherent lymphocytes were removed. We expanded the remaining keratinocytes before performing FISH to select for the live replicating cells that had survived the inflammatory insult. Chromosomes 7, 8, 11, and 17 were examined because these are frequently abnormal in SCC [33]. However, when we examined healthy keratinocyte cultures with allogeneic PBMCs to mimic inflammation and cellular injury at sites of GVHD, we saw an increase in tetraploid cell numbers after 2 weeks of culture and 2 passages (example in Figure 1A; left panel). Tetraploidy could also be visualized when cell nuclei were stained and analyzed by flow cytometry (example seen in Figure 1A; right panel). Aneuploidy for either chromosome 7 or 8 developed after 10 days of culture, whereas tetraploidy could be observed as early as 3 days (Figure 1B). Untreated keratinocytes demonstrated only rare tetraploid cells after expansion in media (Table 1 N 20).

CD8+ Cells Produce More Ploidy Than CD4+ Lymphocytes

To assess whether normal CD4 or CD8 cells were responsible for the changes observed, we separated CD4 and CD8 cells by a magnetic column and cultured each population with keratinocytes obtained from foreskins. Confluent keratinocytes were cultured with 50,000 CD4+ or CD8+ cells. As a control, one aliquot of 100,000 unseparated peripheral blood

Figure 1. Coculture of keratinocytes from healthy foreskins with allogeneic mismatched lymphocytes. (A) When mismatched allogeneic lymphocytes were cocultured with foreskin, keratinocytes for tetraploid cells could be observed in keratinocyte cultures cocultured with allogeneic lymphocytes. An example of a FISH assay utilizing the X/Y probe is seen (left). Arrows point to tetraploid cells. Tetraploid cells are also distinguishable based on DNA content as measured by flow cytometric methods (right). (B) Keratinocytes cocultured with allogeneic were stained with FISH probes for chromosomes 7 and 8. Samples cultured with lymphocytes had significantly more tetraploidy and aneuploidy than samples expanded in the absence of lymphocytes. Tetraploidy appeared by day 3 of culture; aneuploidy was not seen until day 10.
lymphocytes (PBLs) was incubated with cyclosporine (400 ng/mL) before adding the lymphocytes to the flask to block lymphocyte proliferation and activation. Keratinocytes were passaged three times following addition of lymphocytes and harvested two weeks later. CD4 cells were less effective in generation of aneuploid and tetraploid cells compared to CD8 cells. Addition of cyclosporine prevented these chromosomal changes (Figure 2A). Cultures with CD8 cells produced significantly more inflammatory cytokines than those with CD4 cells (Figure 2B).

Inflammation Produced In Vitro by Mismatched Allogeneic Lymphocytes Decreases Keratinocytes Telomere Length

Inflammation and cell death accompanying GVHD results in significant cell mitosis and repair [37]. To determine if an inflammatory insult was associated with decreases in telomere length, we examined the effect of culturing with unseparated PBLs, purified CD4⁺ T cells, or CD8⁺ T cells on keratinocyte telomere length. Telomere length was shortened by the addition of lymphocytes, most significantly after exposure to CD8⁺ cells (Figure 3).

Loss of P53 in Keratinocytes Results from Deletion of Chromosome 17

To assess whether loss of TP53 could account for the frequency of tetraploidy and aneuploidy observed in keratinocyte culture, we stained with a locus-specific probe (LSP) to p53 and a CEP probe to chromosome 11. When tetraploid and aneuploid cells were compared with diploid cells, TP53 gain or loss was significantly greater in aneuploid cells ($P < .0001$) (Figure 4). To determine if TP53 loss resulted from loss of only TP53 or of the entire chromosome 17 (where TP53 resides), we stained cells with an LSP to TP53 and a CEP probe to 17. These experiments indicated that the loss of TP53 was the result of the deletion of chromosome 17 in the majority of cases (data not shown) ($P < .0001$; Fisher’s exact test).

Keratinocytes Exposed to Lymphocytes Demonstrate No p53 Mutation

To determine if keratinocytes exposed to lymphocytes developed mutations in p53, we sequenced the p53 gene. We found no mutations in either the untreated or treated keratinocytes.

DISCUSSION

In this study, we observed tetraploid cells in unmanipulated buccal mucosal smears obtained from sites of GVHD. Even greater numbers of polyploid cells were observed following proliferation of these keratinocytes, suggesting that repeated cell division played a role in alteration of chromosome number. Keratinocytes exposed to allogeneic lymphocytes exhibited some of the chromosomal abnormalities frequently found in patients with SCC [27]: aneuploidy for chromosomes 8, 7, 17, and 11, and tetraploidy. We observed loss of p53 in many of the aneuploid cells—a factor that may be responsible for their continued growth and survival. Telomere length decreased with keratinocyte expansion, but this decrease was more substantial in...
keratinocytes exposed to allogeneic lymphocytes, most notably CD8 cells.

In our experiments, loss of TP53 in most cases appeared to be related to the deletion of chromosome 17- a frequent finding in SCC [27] - rather than to a mutation. Others have observed this nonrandom deletion of chromosome 17 in cells aneuploid for other chromosomes [38]. TP53 triggers apoptosis in aneuploid and tetraploid cells, and haploinsufficiency is associated with survival of aneuploid cells [39]. Tetraploidy has been observed in many premalignant states and may give rise to aneuploid cells [38]. In one proposed model of tumorigenesis in ulcerative colitis (UC) [26], nitric oxide (NO)-induced cell cycle arrest leads to transient tetraploidy and DNA repair. Reactive oxygen species repeatedly exposing TP53 to oxidative stress may result in mutation or loss of heterozygosity of this gene. These events may result in loss of this TP53s checkpoint function and a greater proclivity for malignant transformation. In the TP53 null mouse, tetraploid cells continue to divide but are genomically unstable, acquiring many new translocations or undergoing multipolar mitosis because of duplication of the spindle apparatus, which results in aneuploidy [40].

P53 protein also plays an important role in eliminating cells with telomere dysfunction by triggering senescence or apoptosis in cells with shortened telomeres [41]. Functional loss or gain of TP53 is associated with continued telomere erosion and subsequent formation of dicentric chromosomes resulting from telomeric fusions [42]. Normal cells, adjacent to SCC, reportedly have shortened telomeres, suggesting that reduction of telomere length could be related to malignant transformation [43]. Inflammatory diseases with a proclivity for malignant transformation such as Barrett’s esophagitis [11], ulcerative colitis [44], and hepatitis [45,46], all show substantial decreases in telomere length. Telomere shortening is associated with aneuploidy both in human cancer as well as in the mouse model [47,48]. End-to-end fusion of telomeres with unequal segregation of chromosomes has been observed in the telomerase-deficient mouse and is presumed to be the mechanism for aneuploidy in this circumstance. Epithelial tissue has minimal telomerase activity, so that factors that increase cell turnover would be expected to give rise to more pronounced telomere shortening [49].

These findings suggest that inflammation alone in the absence of immunosuppression may give rise to tetraploid and aneuploid keratinocytes as well as haploinsufficiency of p53. These events may lead to genomic instability, resulting in malignant transformation.

**Figure 3.** Telomere lengths in keratinocytes cultured with lymphocytes. Left panel: keratinocytes were cultured with PBLs as described in Methods and Materials and then passaged two times over the course of 17 days. Telomere length was then measured. PBLs caused a decrease in keratinocyte telomere, which was most prominent immediately following cocultivation with lymphocytes. Right panel: keratinocytes were cultured with 30,000 purified CD4<sup>+</sup>, CD8<sup>+</sup>, and a mixture of similar numbers of CD4 and CD8 cells (ie, 60,000 total lymphocytes), which were preincubated with cyclosporine. CD8<sup>+</sup> cells showed the most dramatic change in telomere length.

**Figure 4.** Loss of TP53 gene following coculture with keratinocytes. An aliquot of lymphocyte-treated keratinocytes was probed with locus specific p53 signal. Cells were probed with CEP 11 and LSP p53. When this was analyzed statistically using the Mann-Whitney test, an excess number of aneuploid and tetraploid cells showed either a gain or loss of p53 (P < .001).
[50]. This in vitro system may provide a model of GVHD with inflammation resulting in DNA damage, loss of TP53, and shortened telomere length—all of which may be steps in the development of SCC.

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AUTHORSHIP STATEMENT

Elaine Sloand designed the study, performed the research, wrote the paper, and analyzed the data. Loretta Pfannes performed the research and wrote the paper. Casey Ling performed the research. Monika Jasek performed the research. Rodrigo Calado performed the research. John Barrett designed the study, analyzed the research. Jaroslaw Maciejewski performed the research. Loretta Pfannes performed the research and wrote the paper, and analyzed the data. Neal Young designed the study and analyzed the data.

REFERENCES


