Little evidence of donor-derived epithelial cells in early digestive acute graft versus host disease.

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Abstract

Donor origin of epithelial intestinal cells has been studied in animals and humans after transplantation and has been used as evidence of hematopoietic stem cell (HSC) plasticity. However, in the human gastro-intestinal tract no study used X and Y-chromosome detection by fluorescence in situ hybridization (FISH) coupled with immunological stainings to characterize cell types on the same tissue section. We here combined these techniques on the same section of duodenal epithelium in 6 patients with acute graft versus host disease. Donor-derived lymphoid cells were detected both in the epithelium and lamina propria, as expected. However, no donor-derived cells could be proven to be of epithelial type, using our stringent criteria.

Introduction

Studies in animal models and hematopoietic stem cell transplant recipients in humans have shown that marrow stem cells could differentiate into other cell types than blood cells. Donor-derived digestive epithelial cells were found in a mouse model and in bone marrow recipients in two human studies. All these studies were based on FISH and immunostaining analyses on tissue sections. However, the concept of HSC plasticity has been questioned by results of experimental studies and it has been suggested that HSC may fuse with other cells and give the appearance of differentiation. We developed technical conditions to reliably address the donor origin and the type of the chimeric cells, and applied this on duodenal biopsies in a series of 6 female recipients of sex-mismatched grafts.

Patients and methods

The 6 female patients received an allogeneic, non T-cell depleted, bone marrow transplant from a male sibling donor. All were grafted for leukemia, and were full donor chimera, without relapse, at the time of biopsy. Duodenal biopsies were performed for diagnostic purpose during endoscopic examination 14 to 33 days
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after the transplant because of diarrhea. Biopsies from female patients grafted with a female donor were considered as negative controls and biopsies from male recipients grafted with a male donor as positive controls. All patients gave their consent to this study according to the Institution’s procedures.

All biopsies were fixed in AFA (alcohol, formalin, acetic acid) and further processed for paraffin embedding. Sister sections of 5 µm thick were realized on each block, the first section being stained with hematoxylin-eosin. The histological diagnosis and the grading of digestive graft versus host disease (GvHD) [table 1] were established according to the criteria of Sale et al, Epstein et al and Washington et al, as previously reported18. The following sister section was used for combined FISH, immunohistochemistry, and immunofluorescent methods. For the X and Y FISH procedure, the different steps of the protocol described by Johnson et al19 were performed but we included immunohistochemistry and immunofluorescent stainings as follows. The first step was removal of paraffin with xylene and rehydratation with alcohol. A lymphoid (CD45) immunoperoxidase staining step was performed before the enzymatic digestion step of the FISH procedure and followed by hematoxylin counterstaining. An epithelial (AE1AE3) fluorescent staining was performed at the end of the FISH process.

Reagents were a CEP X (Spectrum green)/Y (Spectrum orange) DNA probe (Vysis, Downers Grove, Ill, USA), mouse monoclonal anti-CD45 antibody (clone PD7/26 and 2B11, Dako, Carpinteria, California), mouse monoclonal anti-cytokeratin primary antibody (clone AE1AE3, Boehringer Mannheim, Indianapolis) revealed by a second AMCA-conjugated horse anti-mouse IgG antibody (Vector laboratories) whose spectrum is the same as Dapi. The slides were not counterstained by Dapi and were cover-slipped with mounting medium.

The tissue sections were blindly analyzed by two different specialists in pathology and hematology (VM and JS) with a an upright epi-fluorescence microscope (Leica DMR(D), Rueil-Malmaison, France) equiped with 3 appropriate filters blocks for fluorescent analysis (Spectrum green, Spectrum Orange and AMCA) and with bright light. The microscopic pictures were captured through a Plan Apo 63x/1.32 N.A oil immersion objective (0.132 mm/pixel) with a colour tri-CDD
Camera (3x1/2’’) LEI-750D CE system and recorded on a Pentium PC via a Matrox Meteor PCI frame grabber board, using LEICA Q-Win software.

The number of X signals in both the epithelium and the lamina propria and the number of Y signals in the lamina propria were counted on 500 cells. The number of Y signals in the epithelium was counted on 5000 epithelial cells (table 1). Whenever we detected an Y signal in a cell localized in the epithelium, 4 pictures, one in each of the 3 fluorescent spectrum and one in bright light, were captured. The microscopic pictures were matched with the GIMP software to eliminate non-specific signals and to analyze the type of the chimeric cells.

Results and discussion

In positive controls (male recipients from male donors), X and Y signals were respectively 81 versus 73 per 100 cells in the lamina propria and 75 versus 82 per 100 cells in the epithelium. In negative control (female recipients from female donors), we didn’t find any Y-chromosome-specific signal. In female patients grafted from male donors, Y-chromosome specific signals were more numerous (3 to 36 mean 13.2 per cells) in the lamina propria than in the epithelium (0.00 to 0.16 mean 0.053 per 100 cells) (Table 1). By matching microscopic pictures, we demonstrated that 60% to 83%, (mean 69.1%) in the lamina propria and 50% to 100% (mean 91.6%) in the epithelium were of hematopoietic origin as they were CD45 labeled (Figure 1A). These mononuclear cells most probably were lymphocytes or macrophages infiltrating the digestive tract. In the lamina propria, the non CD45 labeled cells could be stromal cells or even myofibroblasts as previously reported. In the epithelium, only two donor-derived, CD45-negative, XY cells (0.04 per 100 cells) were found in a single patient. These two cells, within the epithelium were not of hematopoietic origin since they did not expressed the CD45 marker. However no epithelial staining was detected in these cells, thus questioning about the nature of these cells (Figure 1B).

These results differ from those previously published in human digestive tract after HSC transplantation. These two previous studies suggested that, though
epithelial cells of donor origin were not numerous (0.4-3.6% for Okamoto et al. and, 4-6% for Korbling et al), they were regularly found. These studies were also performed in sexmismatched transplants but technical conditions used for the tissue study were different. We used a X/Y FISH method which allows elimination of most non-specific large spectrum fluorescence. We also combined FISH and immunostainings and analyzed them on the same tissue section to avoid serial consecutive sections which favors localization bias. In Korbling’s et al. paper, successive sections were used, while in Okamoto’s et al. paper Y-chromosome staining alone was used (and not dual X/Y). Finally, early on during acute GvHD of the digestive tract, the patient studied by Korbling et al. (on day 60) and 2 patients studied by Okamoto et al (on day 26) had also few evidence of donor-derived epithelial cells (6% for Korbling and 0.4%, 0.6% for Okamoto). Thus besides technical difficulties that could be overcome with the method we used, there is little evidence from donor-derived epithelial cells early after HSC transplantation. On the other hand, the 2 male cells we found within the duodenal epithelium may indeed represent the first step of cell fusion, a mechanism that seems to be responsible for bone marrow-derived stem cell repair of damaged tissue21.
### Table 1: Immuno-FISH results

<table>
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<tr>
<th>Patients</th>
<th>Days from BMT</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>X signals</td>
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<td></td>
<td>Y CD45+ signals</td>
<td>Y CD45- signals</td>
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<td>81</td>
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</table>

FISH signals were counted as follows: X signals on 500 cells in epithelium and lamina propria, Y signals on 500 cells in lamina propria and on 5000 cells in epithelium. All results included in this table are related to 100 cells.
Figure 1: FISH and immunostainings on the same duodenal sections of 2 female patients, A and B, having received an allogeneic, non T-cell depleted bone marrow transplantation from a male sibling donor. The epithelial basal membrane is underlined by a broken line on all the different pictures.

A1: in the epithelium, presence of a cell with a red signal (white arrow) corresponding to the Y chromosome labeling (FISH x 630). X chromosomes are green stained.

A2: the same cell (white arrow) is not stained with the anticytokeratin antibody (indirect immunofluorescence x 630). Epithelial cells are white stained in a blue background (empty black arrow head).

A3: the same cell (white arrow) is stained with the antiCD45 antibody (indirect immunoperoxidase staining x 630). A lymphoid cell is brown stained in the lamina propria (full black arrow head).

A4: higher magnification of A1 centered on the XY cell in the epithelium (FISH x 3150): X chromosome; green spot. Y chromosome; red spot.

B1: in the epithelium, presence of a cell with a red signal (white arrow) corresponding to the Y chromosome labelling (FISH x 630). X chromosomes are green stained.

B2: the same cell (white arrow) is not stained with the anticytokeratin antibody (indirect immunofluorescent staining x 630).

B3: the same cell (white arrow) is not stained with the antiCD45 antibody (indirect immunoperoxidase staining x 630).

B4: higher magnification of B1 centered on the XY cell in the epithelium (FISH x 3150): X chromosome; green spot. Y chromosome; red spot.
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