Article

Cell-in-cell Structures Formed between Human Cancer Cell Lines and the Cytotoxic Regulatory T-cell Line HOZOT

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We previously established a novel cell line, termed HOZOT, derived from umbilical cord blood mononuclear cells that is characterized as a human cytotoxic regulatory T (Treg) cell line with a FOXP3+CD4−CD8−CD25+ phenotype. Here, we describe a new property of HOZOT cells: they actively penetrate into a variety of human cancer cell lines, but not into normal cell lines, and form apparent cell-in-cell structures. In the process of cell penetration, we observed that HOZOT cells adhered to target cells seemed to first insert their nuclei into the cytoplasm of target cells, distinct from the process of phagocytosis. In addition, blocking experiments showed that major histocompatibility complex class I is one of the target cell recognition molecules for HOZOT cells. Furthermore, we propose that cell-in-cell structures between HOZOT cells and target cancer cells could be one of the cytotoxic mechanisms of HOZOT cells.

Keywords: cell-in-cell, emperipolesis, HOZOT, regulatory T cell

Introduction

A unique and intriguing phenomenon, termed cell-in-cell structures, in which cells are found in the cytoplasm of other cells, has recently received much attention (Overholtzer and Brugge, 2008). One type of cell-in-cell structure, in which phagocytic cells engulf dead, dying or pathogenic cells, is termed ‘phagocytosis’, and phagocytosis theory is generally recognized as the first pivotal line of host defense (Chernyak and Tauber, 1988; Henson and Hume, 2006; Ravichandran and Lorenz, 2007). On the other hand, in 1956, Humble et al. (1956) introduced the new term ‘emperipolesis’ to describe the phenomenon distinct from phagocytosis whereby lymphoid cells apparently penetrate into the cytoplasm of a variety of host cells. In addition, another novel cell-in-cell structure was described as ‘entosis’ during which extracellular matrix detachment can provoke homotypic cell-in-cell structures in various epithelial cancer cell lines (Overholtzer et al., 2007). These results suggest that live cells could homotypically or heterotypically penetrate into other cells under certain conditions. Many reports describing phagocytosis, emperipolesis and entosis were published from each point of view. Nevertheless, especially in emperipolesis, cell recognition, penetration mechanisms and their physiological roles remain unexplained. Insights from detailed studies of cell-in-cell structures formed by lymphocytes and host cells may be crucial to better understanding emperipolesis.

Regulatory T cells (Tregs) are a subset of T lymphocytes that play a central role in inducing and maintaining immune tolerance and in the termination of immune responses. Deficiency or dysfunction of these cells leads to autoimmunity or enhanced pathogen-induced inflammation (Maggi et al., 2005; Sakaguchi, 2005). Several distinct types of Tregs are distinguished by their phenotype, cytokine production and function (Roncarolo et al., 2001; Weiner, 2001; Jiang and Chess, 2004). CD4+CD25+ Tregs are a well-documented subpopulation of Tregs and the importance of CD4+CD25+ Tregs has been confirmed in many types of autoimmune diseases (Shevach, 2002; Sakaguchi, 2004).

We recently characterized a novel human Treg cell line established from umbilical cord blood (UCB) mononuclear cells (MNCs) by coculture with mouse stromal cell lines. This cell line, termed HOZOT, is characterized as consisting of cytotoxic Treg cells with a FOXP3+CD4−CD8−CD25+ phenotype. In brief, HOZOT is characterized by the following criteria: (i) having cytotoxic activity against stromal cells, (ii) containing more than 20% of CD4+CD8− cells, (iii) suppressing the proliferation induced by allo-MLR, (iv) producing IL-10, (v) exhibiting anergy following stimulation with anti-CD3/CD28 antibodies (Nakamura 2004).
et al., 2007; Suzuki et al., 2009). Moreover, HOZOT cells exhibit cytotoxic activity against several human cancer cell lines (e.g. WiDr, ZR-75-1 and MKN-45), but not normal human cell line (NHDF). Cytotoxic T lymphocytes (CTLs) play a critical role in the immune system. They are able to recognize and destroy virally infected and tumorigenic cells by secreting granzymes and perforin that are packaged in cytoplasmic granules (Stinchcombe and Griffiths, 2007). Although HOZOT cells include cytotoxic granules (e.g. granules containing granzymes and perforin) in their cytoplasm, exocytosis inhibitors do not inhibit the cytotoxicity of HOZOT cells against target cancer cells. Moreover, we confirmed that TNF-α, IFN-γ and Fas molecules are not involved in the cytotoxic activity of HOZOT cells. These results suggest that HOZOT cells have killing mechanisms other than the secretion of cytotoxic granules, which act from outside the target cells. In the course of studying the killing mechanism(s) of HOZOT cells, we observed the phenomenon that HOZOT cells actively penetrate into target cancer cells and form cell-in-cell structures, which we categorize as emperipolesis. Furthermore, the formation of cell-in-cell structures may be the mechanism by which HOZOT cells exert cytotoxicity against cancer cell lines.

**Results**

**HOZOT cells are similar to large granular lymphocytes**

To study the cytotoxic properties of HOZOT, we utilized WiDr, a human colorectal adenocarcinoma cell line that is effectively killed by HOZOT cells, as targets. We observed atypical cells with morphology distinct from either HOZOT or WiDr cells when HOZOT cells were added to a culture of WiDr. We then performed detailed morphological examinations of these atypical cells.

As a first step, we carefully examined the morphology of HOZOT cells. Cytospins of HOZOT cells were stained with Wright’s solution and analyzed by optical microscopy. HOZOT cells had well-developed cytoplasms, and Wright’s stain-positive granules were detected in their cytoplasms (Figure 1A). Similar to CTLs, immunofluorescent microscopy revealed granzyme B-, perforin- and RANTES-positive granules in the cytoplasms of HOZOT cells (Figure 1B–D). Electron microscopy revealed a hand-mirror shape of HOZOT cells with the nucleus located at the leading edge. In addition, dispersed electron dense granules and a swollen Golgi apparatus were found in well-developed cytoplasms of HOZOT cells (Figure 1E). These morphological observations revealed that HOZOT cells have morphological properties similar to activated large granular lymphocyte cells.

**Cell-in-cell structures are formed between HOZOT and WiDr cells**

Our next step was to perform similar morphological analyses on cocultured cells. Cytospins obtained from cocultures of HOZOT and WiDr were examined by optical microscopy. We observed atypical cells that appeared to have two or three nuclei in a single cell. Morphological observation indicated that the atypical cells were WiDr cells (with a larger cell size, basophilic cytoplasmic staining, and a bigger and denser nucleus) penetrated by HOZOT cells (with a smaller cell size, lighter staining nucleus and Wright’s stain-positive granules) (Figure 1F). This observation reminded us of cell-in-cell structures reported in a study of epithelial cancer cell lines (Overholtzer et al., 2007). In the cell-in-cell structures consisting of HOZOT and WiDr cells, the nuclei of WiDr cells were compressed into a semicircular shape by internalized HOZOT cells. Occasionally, a HOZOT cell penetrated into the mitosis phase of a dividing WiDr cell, compressing the chromosomes of the WiDr cell (Figure 1G).

To confirm the observation that HOZOT cells actually penetrate into WiDr cells, each cell population was labeled separately with red (HOZOT) and green (WiDr) fluorescent CellTracker dyes, and then mixed together in cell culture. Internalized cells were observed as early as 2 h after initiating coculture. Fluorescent microscopy demonstrated that red (HOZOT) cells resided within the cytoplasm of green (WiDr) cells (Figure 1H). Moreover, three-dimensional analysis of confocal laser microscopy demonstrated the complete internalization of HOZOT (red) cells within WiDr (green) cells (Figure 1).

**Electron microscopic examination of cell-in-cell structures**

To further examine the detailed cell-in-cell structures, cocultured HOZOT and WiDr cells were examined by electron microscopy. Electron lucent ‘halo’ structures were observed between WiDr cytoplasms and the plasma membranes of HOZOT cells, indicating that these intracellular HOZOT cells exist within a cytoplasmic vacuole derived from the WiDr cell (Figure 2A). Ultrastructural examination also revealed evidence of dead or dying features associated with HOZOT cells within WiDr cells. Internalized HOZOT cells within WiDr cells exhibited degraded cytoplasm, electron lucent structure of nucleus, increased number of vacuole-like structure and ruptured cell membrane of HOZOT cells were observed. On the other hand, WiDr cells including HOZOT cells demonstrated numerous swollen rough endoplasmic reticula in the cytoplasm, enlarged nucleolus, aggregated chromatin on the nuclear membrane and homogenous euchromatin of nucleus (Figure 2B). Nevertheless, dead or dying HOZOT cells within dead WiDr cells were rarely observed in 2 h cocultures.

**HOZOT cells are surrounded by β-catenin-positive membranes derived from WiDr cells**

Our examinations up to this point suggest two possibilities. The first is that internalized HOZOT cells are surrounded by the plasma membrane of WiDr cells in vacuole-like structures during emperipolesis. The other is that HOZOT cells broke through the plasma membrane of WiDr cells and penetrated into the target cells through the broken area. To distinguish between these possibilities, cytospun samples were stained with anti-β-catenin antibody. Optical microscopy demonstrated that pale-colored cytoplasmic areas of internalized HOZOT cells
Figure 1 Morphology of HOZOT cells and cell-in-cell structures formed between HOZOT and WiDr cells. (A) Optical micrograph of HOZOT cells stained with Wright’s solution. The developed cytoplasm of HOZOT cells were stained pale blue, and both dispersed granules (arrows) and nuclei (arrowheads) were stained dark violet (bar indicates 10 μm). (B–D) Immunofluorescent micrographs of HOZOT cells stained with anti-human granzyme B, anti-perforin and anti-RANTES. Immuno-reactive granules (green) containing, (B) granzyme B, (C) perforin and (D) RANTES were detected in the cytoplasm of HOZOT cells (bar indicates 10 μm). (E) A representative electron micrograph of HOZOT cells. A HOZOT cell showing a hand-mirror shape and containing many electron dense granules (arrows) and a swollen Golgi apparatus (arrowheads) in a well-developed cytoplasm (bar indicates 1 μm). (F) Optical micrographs of cell-in-cell structures between HOZOT and WiDr cells stained with Wright’s solution. Internalized HOZOT cells (arrowheads) within WiDr cells showing the compressed semicircular nuclei of WiDr cells. Wright’s stain-positive granules were detected in the cytoplasm of internalized HOZOTs (arrow) (bar indicates 20 μm). (G) Occasionally, we observed internalized HOZOT cells (arrowhead) inside of WiDr cells with compressed chromosomes (arrow) (bar indicates 20 μm). (H) Fluorescent micrograph of cocultured CellTracker-stained HOZOT (red) and WiDr (green) cells. HOZOT (red) cells were detected in the cytoplasm of WiDr (green) cells (bar indicates 10 μm). (I) Confocal laser microscopy showing HOZOT (red) cells completely embedded within WiDr (green) cells (see the red cell under the cross point of the black lines) (bar indicates 10 μm).
were separated from the cytoplasm of WiDr cells by a dark-colored boundary (Figure 2C). This boundary line between the cytoplasm of internalized HOZOT cells and the cytoplasm of WiDr cells seems to be formed by the cell membrane of WiDr cells. Beta-catenin expression can distinguish positive epithelial cells from negative T lymphocytes. In addition to the outer peripheral area of WiDr cells, β-catenin-positive fluorescence was also observed within WiDr’s cytoplasm as a ring-like structure encircling the nucleus of internalized HOZOT cells (Figure 2D and E). These results and electron microscopic observation revealed that HOZOT cells within WiDr cells are surrounded by the plasma membrane of WiDr cells in vacuole-like structures.

Kinetics of forming cell-in-cell structures

To investigate the mechanism of the formation of cell-in-cell structures, we first established a culture system to determine the frequency of cell-in-cell structures in a quantitative manner. A HOZOT:WiDr (effector:target) ratio of 5:1 was used for further analysis because this ratio has been used as a minimum effective ratio for cytotoxic studies in our laboratory. To determine an optimal culture time, we studied the kinetics of forming cell-in-cell structures. Cocultures of HOZOT and WiDr cells were monitored at 2, 4 and 8 h. When cultured alone, WiDr cells did not detach from the bottoms of culture plates. Nevertheless,
when cocultured with HOZOT cells, adhered WiDr cells started to detach from the bottoms of culture plates after incubation for 2 h. Therefore, to quantify the frequency of cell internalization, both detached and adherent WiDr cells were separately collected and cytospun onto glass slides at each time point. The number of WiDr cells that include HOZOT cells was counted and calculated as the cell-in-cell index (%) representing the frequency of cell internalization. Both detached and adherent WiDr cells reached the maximum frequency of internalized HOZOT cells at 4 h of coculture. Therefore, an incubation period of 4 h at 37°C was selected as the optimum culture time for routine use (Figure 3A).

HOZOT cells form cell-in-cell structures with a variety of cancer cell lines

We next investigated target cell specificity by coculturing five human cancer cell lines (WiDr, ZR-75-1, MKN-45, LoVo and HepG2) and three human normal cell lines (NHDF, NHEK and HUVEC) with HOZOT cells. HOZOT cells formed cell-in-cell structures with each of the five cancer cell lines, whereas very few cell-in-cell structures were observed in the cocultures with the three normal cell lines (<1%) (Figure 3B). These results suggest that HOZOT cells may selectively form cell-in-cell structures with cancer cells. In the cancer cell lines investigated, the frequencies of cell-in-cell structures in MKN-45 (gastric adenocarcinoma) and ZR-75-1 (mammary ductal carcinoma) were higher than those in the other three cancer cell lines. Hereafter, MKN-45 cells were used as a representative cell line for our routine study, because they are the most sensitive target cells and gastric cancer cells develop in both females and males.

HOZOT cells actively penetrate into target cells

In general, phagocytosis is driven by cytoskeletal rearrangements in the engulfing cells. To examine whether HOZOT cells actively penetrate into MKN-45 cells or MKN-45 cells engulf HOZOT cells through phagocytic activity, HOZOT or MKN-45 cells were individually treated with 0.2 or 2 μM of cytochalasin E, an inhibitor of actin polymerization, for 2 h at 37°C. Treatment of MKN-45 cells with cytochalasin did not inhibit the cell internalization of HOZOT cells, whereas cell internalization of HOZOT cells was decreased by treating HOZOT cells with cytochalasin in a dose-dependent manner (Figure 4A). In the cocultured HOZOT and MKN-45 cells, phase-contrast microscopy revealed that hand-mirror-shaped HOZOT cells seemed to attach and closely adhere to MKN-45 cells (Figure 4C and D). In addition, optical microscopy demonstrated that the adhered HOZOT cells seemed to first insert its nucleus into the cytoplasm of the target cell (Figure 4E–H). In this process, the nuclei of HOZOT cells adopted a dumbbell shape, and the cytoplasm of the HOZOT cell seemed to remain outside the MKN-45 cell (Figure 4H and I). These results revealed that HOZOT cells actively penetrate into the cytoplasm of target cancer cells. Hereafter, positive cell internalization is referred to as cell-in-cell activity. Because detached MKN-45 cells showed a higher frequency of cell-in-cell structures than adhered cells, we show only the results for detached cells with regard to cell-in-cell activity in our subsequent experiments.

Other kinds of activated T cells do not form cell-in-cell structures

HOZOT cells can be regarded as activated Tregs with a FOXP3+CD4+CD8−CD25+ phenotype, because they were induced by stimulation with mouse stromal cell lines. To compare cell-in-cell activity among other kinds of activated T cells, in vitro-induced Treg (iTreg) cells or anti-CD3/CD28

Figure 3 Kinetics of the formation of cell-in-cell structures with a variety of cancer cell lines. (A) Kinetics of the formation of cell-in-cell structures between HOZOT and WiDr cells. Cell-in-cell structures were counted and the frequency of cells internalization was quantified at 2, 4 and 8 h after incubation. Maximum frequency of cell-in-cell structures was observed at 4 h after cocultivation. Results represent the mean of ± SD for each cytospin (n = 4, **P < 0.01). (B) Five human cancer cell lines (ZR-75-1, MKN-45, LoVo, HepG2 and WiDr) and three human normal cell lines (HUVEC, NHDF and NHEK) were cocultured with HOZOT cells. After 4 h, detached and adherent cells were collected, cytospun and cell-in-cell structures were counted. HOZOT cells showed the highest frequency of cell internalization with ZR-75-1 and MKN-45, whereas HOZOT cells rarely penetrated into the three normal cell lines. Results represent the mean of ± SD for each cytospin (n = 4).
antibody-stimulated T (Con T) cells were cocultured with MKN-45 cells. Induced Treg (iTreg) cells were used as representative Treg cells and Con T cells were used as representative effector T cells. No cell-in-cell activity was observed when using iTreg and Con T cells cocultured with MKN-45 cells (Figure 5A). Since HOZOT cells exhibit some NK markers, NK-92 (human NK cell line) cells were used as representative NK cells, whereas JURKAT (human leukemic T-cell line) cells were used as general activated T cells. NK-92 and JURKAT cells were also unable to form cell-in-cell structures when cocultured with MKN-45 cells (Figure 5B). Furthermore, IL-2 dependency of NK-92 cells was not observed in our culture systems (Supplemental Figure S1). These results demonstrate that only HOZOT cells exhibit cell-in-cell activity among a number of different activated T cells.
Adhesion molecules assist the formation of cell-in-cell structures

Because lymphocytes recognize cell adhesion molecules expressed on endothelial cells, we next examined whether HOZOT cell recognition of certain cell adhesion molecules, including ICAM-1, CD62L and LFA-2, is required to penetrate into target cancer cells. Anti-ICAM-1 (10 µg/ml) and anti-CD62L (10 µg/ml) antibodies exhibited partial inhibition on the cell-in-cell activity of HOZOT cells. However, anti-LFA-2 (10 µg/ml) did not inhibit the cell-in-cell activity of HOZOT cells (Figure 6A). These results suggest that ICAM-1 seems to be one of the molecules recognized by HOZOT cells, but not a primary molecule for target recognition. In addition, anti-CD62L also resulted in partial inhibition of the cell-in-cell activity of HOZOT cells. Since WiDr, MKN-45 and ZR-75-1 cells express ligands of CD62L (e.g. sialyl Lewis A and sialyl Lewis Y) (Dippold et al., 1987; Sato et al., 1997), we added anti-sLewis A (10 µg/ml) or anti-sLewis Y (10 µg/ml)
antibodies into cocultures of HOZOT and MKN-45 cells. Anti-sLewis A antibody partially inhibited the cell-in-cell activity of HOZOT cells, but anti-sLewis Y antibody did not (Figure 6B). These results indicate that sLewis A may be recognized by HOZOT cells, but this may not be the primary molecule for target recognition.

Major histocompatibility complex class I/CD8 interactions help form cell-in-cell structures

Since CTL recognize target cells through the interaction of major histocompatibility complex (MHC) class I and TCR/CD8 molecules, anti-MHC class I (10 μg/ml) and anti-CD8 (10 μg/ml) antibodies were used to inhibit the cell-in-cell activity of HOZOT cells. Both anti-MHC class I and anti-CD8 antibodies inhibited the cell-in-cell activity of HOZOT cells (anti-MHC class I; 31% inhibition and anti-CD8; 29% inhibition). Furthermore, mixing these antibodies resulted in greater inhibition than single-antibody treatments (mixture; 51% inhibition) (Figure 6C). Therefore, MHC class I seemed to be the primary molecule used by HOZOT cells to recognize target cells.

Internalized TUNEL or anti-cleaved caspase-3-positive HOZOT cells negatively impact target cell viability

In Figure 2B, electron microscopic examination demonstrated a possibility that dead or dying HOZOT cells within the target cells may exert a cytotoxic effect from inside the target cells. Therefore, to determine the relevance of TUNEL or anti-cleaved caspase-3-positive HOZOT cells within MKN-45 cells, we next examined relationship between apoptotic HOZOT cells and viability of MKN-45 cells. Cocultures of HOZOT and MKN-45 cells were incubated at 37°C for 4 and 8 h. At the indicated time points, detached cells were collected and the frequencies of TUNEL or anti-cleaved caspase-3-positive HOZOT cells in MKN-45 cells were measured. At 4 h incubation, MKN-45 cells cocultured with HOZOT cells exhibited high cell viability (Figure 7A), and TUNEL or anti-cleaved caspase-3-positive HOZOT cells within MKN-45 cells were occasionally observed (Figure 7B and D). A rare case of a pair of HOZOT cells, one alive and the other TUNEL positive, was observed within an alive MKN-45 cell (Figure 7C). The frequencies of TUNEL or anti-cleaved caspase-3-positive HOZOT cells within MKN-45 cells increased over time, whereas the cell viability of the MKN-45 cells inversely decreased (Figure 7E). In addition, MKN-45 cells cocultured with cytochalasin-treated HOZOT cells exhibited high cell viability and a decreased frequency of cell internalization (Figure 4A and 7E). These results demonstrate that MKN-45 cells including TUNEL or anti-cleaved caspase-3-positive HOZOT cells may die following the cell death of penetrated HOZOT cells. Next, to see whether TUNEL or anti-cleaved caspase-3-positive HOZOT cells exert a cytotoxic effect from inside MKN-45 cells, we performed blocking study with apoptosis inhibitor (caspase-3 inhibitor). Efficacy of caspase-3 inhibitor was confirmed by preliminary examination with anti-Fas monoclonal antibody (Supplemental Figure S2). Caspase-3 inhibitor-treated HOZOT cells were cocultured with MKN-45 cells at 37°C for 8 h. The frequency of TUNEL-positive HOZOT cells within MKN-45 cells significantly decreased (Figure 7F), whereas the cell viability of MKN-45 cells inversely increased (Figure 7G). The blocking effect of caspase-3 inhibitor was not complete, indicating the involvement of other unknown cytotoxic mechanisms. These results suggest that dead HOZOT cells within the MKN-45 cells may exert a cytotoxic effect from inside the target cells, providing a clue to understanding one of the cytotoxic mechanisms of HOZOT cells.

Discussion

We report in this study that HOZOT cells, previously characterized as cytotoxic Tregs, actively penetrate into target cancer cells. Microscopic observations suggest that HOZOT cells first attach to the target cells and insert their nuclei into the cytoplasm of target cells, forming the so-called cell-in-cell structures (Figure 4E–H). This phenomenon exhibited by HOZOT cells can be categorized as emperiplois. In emperiplois, a number of reports described lymphocytes penetrating into host cells. Burns et al. (1981, 1982) documented that Fc receptor-positive lymphocytes or non-T cells exhibited penetration into host cells. Nevertheless, definite cellular characteristics of these penetrating lymphocytes remained unexplained to date. Our study is the first report of a well-characterized Treg cell line actively penetrating into target cancer cells.

When lymphocytes bind to endothelial cells or tissues, cell adhesion molecules on these cells are recognized by lymphocytes. Tanaka et al. (1997) reported that antibodies to LFA-1/ICAM-1 inhibit emperiplois of lymphocytes. In our experiments, anti-ICAM-1 and anti-CD62L antibodies exhibited partial inhibition on the cell-in-cell activity of HOZOT cells (Figure 6A). HOZOT cells express ICAM-1, whereas WiDr, MKN-45 and ZR-75-1 cells do not express the ligands of ICAM-1 such as LFA-1 and integrin β2. Conversely, HOZOT cells express LFA-1 and integrin β2; however, WiDr, MKN-45 and ZR-75-1 do not express ICAM-1 and ICAM-2 (our unpublished data). These results suggest that other unknown molecules could be involved in ICAM-1-mediated recognition mechanisms between HOZOT and target cells. Nevertheless, ICAM-1 does not seem to be the main molecule for target recognition of HOZOT cells, since anti-ICAM-1 exhibited only partial inhibition on the cell-in-cell activity of HOZOT cells. In addition, HOZOT cells express CD62L, and WiDr, MKN-45 and ZR-75-1 express CD15s (sLewis X) and sLewis A, which are ligands of CD62L (Dippold et al., 1987; Sato et al., 1997). Nevertheless, anti-CD62L and anti-sLewis A antibodies exhibited only partial inhibition on the cell-in-cell activity of HOZOT cells. Altogether, these results indicate that these adhesion molecules, performing cell contact between HOZOT and target cells, are not the primary molecules for target cell recognition of HOZOT cells.

Cytotoxic T lymphocyte recognition of MHC class I molecules on the target cell surface is necessary for target cell lysis (O’Rourke and Mescher, 1992; Shen et al., 1996). In our study, the cell-in-cell activity of HOZOT cells was inhibited by anti-MHC class I and anti-CD8 antibodies (Figure 6C). These results revealed that HOZOT cells appear to recognize MHC class I molecules,
Figure 7 Inverse correlation between TUNEL or anti-cleaved caspase-3-positive HOZOT cells and target cell viability. (A) TUNEL-positive HOZOT cells within MKN-45 cells was detected with an apoptosis detection kit. Control micrograph of cocultured HOZOT and MKN-45 cells stained for apoptosis. TUNEL-positive reaction is not detected in the nucleus of HOZOT cell (bar indicates 10 μm). (B) After 4 h, the dark brown nucleus (TUNEL positive) of a HOZOT cell is observed in the cytoplasm of an MKN-45 cell (bar indicates 10 μm). (C) A rare case of a pair of HOZOT cells, one alive and the other TUNEL positive, was observed within an MKN-45 cell (bar indicates 10 μm). (D) Cocultured unlabeled HOZOT cells and CellTracker-labeled MKN-45 (red) cells were cytospun, and stained with anti-cleaved caspase-3 antibody. Before cocultivation, nuclei of MKN-45 cells were pre-labeled with Hoechst 33342. Anti-cleaved caspase-3-positive immunofluorescence (green) was observed in the nucleus of the HOZOT cell within the cytoplasm of MKN-45 (red) cell. Hoechst 33342-positive nucleus (pale blue) of the MKN-45 cell exhibited intact structure (bar indicates 10 μm). (E) Frequencies of TUNEL or anti-cleaved caspase-3-positive HOZOT cells within MKN-45 cells, and cell viability of MKN-45 cells. The frequencies of TUNEL or anti-cleaved caspase-3-positive HOZOT cells within MKN-45 cells increased over time, whereas the cell viability of the MKN-45 cells decreased. Results represent the mean of ± SD for each cytospin (n = 4, **P < 0.01). (F) HOZOT cells were pretreated with caspase-3 inhibitor for 1 h at 37°C, and caspase-3 inhibitor-treated HOZOT cells were cocultured with MKN-45 cells for 8 h. Caspase-3 inhibitor-treated HOZOT cells exhibited a decreased frequency of TUNEL-positive cells within MKN-45 cells in a dose of 50 μM. Results represent the mean of ± SD for each cytospin (n = 4, **P < 0.01). (G) Caspase-3 inhibitor-treated HOZOT cells were cocultured with MKN-45 cells for 8 h. The cell viability of MKN-45 inversely increased. Results represent the mean of ± SD for each treatment (n = 4, *P < 0.05).
stimulate cell-in-cell activity of HOZOT cells, in a manner similar as CTLs. Nevertheless, distinct from the process of cell penetration exhibited by HOZOT cells, CTLs first form immunological synapses with target cells and then destroy the targets by secreting cytotoxic granules over a period of a few minutes (Stinchcombe et al., 2001). Interestingly, Rodošević et al. (1995) observed that a number of NK cells penetrated into the cytoplasm of target K-562 cells, concurrently exerting cytotoxic activity. Although this observation suggests a possible relationship between HOZOT and NK cells, HOZOT cells do not show cytotoxic activity against K-562 cells (our unpublished data). These results demonstrate that HOZOT cells seem to be a different cell type from CTL and NK cells.

HOZOT is characterized as a Treg cell line, although HOZOT cells have a CD4/CD8 double-positive phenotype. CD4/CD8 double-positive lymphocytes with Treg properties are present in the intestine as intraepithelial lymphocytes (Das et al., 2003), and these intestinal double-positive Tregs are considered to regulate homeostasis in the intestinal environment (Tsujii, 2006). To date, it is poorly understood whether cell-in-cell structures exist in the intestine (Andrew and Collings, 1946; Dobbins, 1986). Our studies have determined that iTregs do not exert cell-in-cell activity (Figure 5A).

T-cell-mediated immune responses require the active migration of T cells in tumors and the establishment of specific cell–cell contacts. During antitumor response, differentiated T cells (e.g. CD8+ T, NK, γδT and Tregs) migrate in tumors to interact with target cells. In most cases, tumor rejection is initiated by CD8+ CTLs, which infiltrate solid tumors, recognize tumor antigen and kill tumor cells. Recently, tumor infiltrating FOXP3+ CD8+CD25+ T cells (Tβreg), which have strong suppressive capacities, were found in blood and colorectal cancer (Chaput et al., 2009). On the other hand, Bagot et al. (1998) reported that CD4+CD8dim+ T cells, which have cytotoxic activity toward autologous tumor cells, were isolated from cutaneous T cell lymphoma. NK cells (NKGD2+CD8+ or CD4+ T cells) are also a relevant T-cell subtype for immunosurveillance of tumors (Maccalli et al., 2009). Previously, a few reports demonstrated that Tregs have cytotoxic activity (Kemper et al., 2003; Grossman et al., 2004). HOZOT cells are characterized as cytotoxic T cells with a FOXP3+CD4+CD8+CD25+ phenotype, and express NKGD2 molecule. These phenotypical features suggest that HOZOT cells seem to be a new type of Treg having both suppressive and cytotoxic activities.

IL-8 is widely expressed in tumor, stroma and endothelial cells. Its autocrine and paracrine function has been shown to play an important role in angiogenesis, tumor growth and metastasis. IL-8 stimulates T lymphocyte chemotaxis (Jinquan et al., 1995; Xu et al., 1995), and cytotoxic CD8+ T cell has the migration capacity to IL-8 (Hess et al., 2004). These results suggest a possibility that IL-8 produced by MKN-45 cells may induce cell-in-cell activity of HOZOT cells. To clarify the relevance of cell-in-cell activity of HOZOT cells and IL-8 in our culture system, we measured the amount of IL-8 in culture medium of MKN-45 cells. In our assay system, the amount of IL-8 was under the detection limit. Furthermore, Kitadai et al. (2000) reported that MKN-45 cells do not express mRNA of IL-8. Since HOZOT cells penetrate into the target cells without IL-8 in our culture system, cell-in-cell activity of HOZOT cells may be unrelated to IL-8.

In emperipolesis, Okuyama et al. (1979) observed that cancer cells in stomach were killed by lymphocytes that penetrated into the cytoplasm of the cancer cells. In our studies, HOZOT cells penetrated into cancer cells, but not into normal cells (Figure 3B). Cytotoxic granules (e.g. granzyme B and perforin) were identified in the cytoplasm of HOZOT cells (Figure 1B and C). In addition, dead or dying cancer cells that had incorporated HOZOT cells were detected by electron microscopy (Figure 2B). Furthermore, the number of TUNEL or anti-cleaved caspase-3-positive HOZOT cells within target cancer cells increased in a time-dependent manner, whereas the cell viabilities of target cancer cells showed an inverse decrease (Figure 7E). In addition, MKN-45 cells cocultured with cytchalasin-treated HOZOT cells exhibited high cell viability, and the cell viability of MKN-45 cells was rescued by caspase-3 inhibitor treatment of HOZOT cells (Figure 7F and G). Since an in vivo counterpart of HOZOT cells has not been identified in human or mouse, the physiological roles of the cell-in-cell activity of HOZOT cells are difficult to speculate upon. Nevertheless, these results suggest that the cytotoxic granules of penetrating HOZOT cells may be dispersed in the cytoplasm of target cancer cells, thus mediating the killing activity of HOZOT cells from inside the cancer cells, like a Trojan horse.

One molecular mechanism that has been reported to be important for cell penetration during entosis is the Rho-ROCK-actin/myosin pathway (Overholtzer et al., 2007). In our study, cytchalasin, an inhibitor of actin polymerization, inhibited the cell-in-cell activity of HOZOT cells (Figure 4A). This result suggests that the Rho-ROCK-actin/myosin pathway may play a pivotal role in the cell-in-cell activity of HOZOT cells. On the other hand, in the process of cell penetration, we observed the intriguing phenomenon that HOZOT cells first insert their nuclei into the cytoplasm of target cells (Figure 4E–H). Nonetheless, detailed molecular mechanisms of this phenomenon remain unclear. Therefore, clarifying the physiological roles of emperipolesis and the cytotoxic mechanisms of HOZOT cells will require further detailed studies with a particular focus on the molecular mechanisms of HOZOT cell penetration.

Materials and methods

Generation of HOZOT and other types of T cells from UCB

All T-cell lines used in this study were generated from MNCs from UCB. UCB was obtained from Kurashiki Medical Center in compliance with the Institutional Review Board and with informed consent of the donors according to the Declaration of Helsinki. Mononuclear cells were prepared by gradient centrifugation using Ficoll-Paque (GE Healthcare, Buckinghamshire, UK).

The human Treg line, HOZOT, was generated by coculturing UCB MNC with murine stromal cell lines, as reported previously (Nakamura et al., 2007). Briefly, to generate HOZOT cells, CD34− MNCs were enriched by negative selection using an
MACS CD34<sup>+</sup> isolation kit (Milteny-Biotec, Bergisch Gladbach, Germany) and a mini-MACS column, according to the manufacturer’s instructions. The cells were cultured over stromal cells in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, South Logan, UT, USA), 100 U/ml penicillin and 50 mg/ml streptomycin at 37°C in 5% CO<sub>2</sub>. HOZOT cells were established by coculture with the murine stromal cell line, ST2. Once established as a cell line, HOZOT cells were expanded in medium containing 10 ng/ml of IL-2 (Peprotec, EC, London, UK). HOZOT cells were purified by Ficol-Faque to deplete debris from mouse stromal cells killed by HOZOT cells before experiments. Conventional T (con T) cells were obtained by at least 1 week of cultivation of CD4<sup>+</sup>CD25<sup>+</sup> cells on plates coated with anti-human CD3 and anti-CD28 antibodies in the presence of 10 ng/ml of IL-2. The in vitro method for inducing iTregs was reported previously (Godfrey et al., 2005).

Cell lines and cell culture

WiDr (human colorectal adenocarcinoma cell line, ATCC CCL-218), MKN-45 (human gastric adenocarcinoma, RCB1001, RIKEN BioResource Center, Ibaraki, Japan), ZR-75-1 (human mammary ductal carcinoma, ATCC CRL-1500), LoVo (human colorectal adenocarcinoma, ATCC CCL-229), HepG2 (human hepatocellular carcinoma, ATCC HB-8065), NK-2 (human malignant non-Hodgkin’s lymphoma, provided by Dr. H.G. Klingemann, Rush Medical College, Chicago, IL, USA), JURKAT (human acute T cell leukaemia, ATCC TBL-152) and NHDF (normal human dermal fibroblast, KF-4009, Kurabo, Osaka, Japan) were maintained in RPMI 1640 medium supplemented with 10% FCS. HUVEC (normal human umbilical vein endothelial cells, KE-6109, Kurabo) were maintained in HuMedia-EG medium (Kurabo). NHEK (normal human epidermal keratinocyte, KK-4001, Kurabo) were maintained in EpiLife–KG2 medium (Kurabo).

Antibodies and reagents

The following antibodies and reagents were used for functional blocking and immunofluorescent microscopy: anti-human HLA-A,B,C (clone: W6/32, BioLegend, San Diego, CA, USA), anti-human CD2 (clone: SFC13P12H9) and anti-human CD62L (clone: SFC128T17G6, Beckman Coulter, Fullerton, CA, USA), anti-human CD8 (clone: RPA-T8, BD Biosciences, San Jose, CA, USA), anti-sialyl Lewis A (clone: 2D3, Seikagaku Corp., Tokyo, Japan), anti-β-catenin (clone: 14, Transduction Laboratories, Lexington, KY, USA), anti-human ICAM-1 (clone: BBIG-11, anti-human RANTES (clone: 21445), anti-human CD3ε (clone: UCHT1), anti-human CD28 (clone: 37407.111, R&D Systems Inc., Minneapolis, MN, USA), anti-human granzyme B (clone: GrB-7, Kamiya Biomedical Co., Seattle, WA, USA), anti-human perforin (clone: deltaG9, Ancell Co., Bayport, MN, USA), anti-cleaved caspase-3 (Calbiochem, Darmstadt, Germany), anti-Fas (clone: CH-11, Medical & Biological Laboratories, Nagoya, Japan), mouse monoclonal isotype control (clone: MOPC-21, Abcam, Inc., Cambridge, MA, USA), Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG, CellTracker (CMFDA, CMTPX, Invitrogen, Paisley, UK), cytochalasin E (EMD Chemicals, Inc., Darmstadt, Germany) and caspase-3 inhibitor (Ac-DMQD-CHO, Calbiochem, La Jolla, CA, USA). Anti-sialyl Lewis Y monoclonal antibody was prepared in our laboratory (Suzuki et al., 1997).

Electron microscopy

For electron microscopy, cells were fixed in a mixture of 1% glutaraldehyde (TAAB Laboratories Equipment Ltd., Berks, UK) and 2% paraformaldehyde (Merck KgaA, Darmstadt, Germany) in 0.1 M PBS (pH 7.4) for 2 h at 4°C and post fixed in 1% osmium tetroxide (Merck KgaA) in 0.1% PBS. After dehydration through an ethanol series, the specimens were embedded in Araldite-Epon (Nissin EM, Tokyo, Japan). Ultrathin sections were prepared with a Reichert-Jung Ultracut Omlu4 ultramicrotome (Reichert-Jung, Inc., Austria). Pale-gold sections were collected on 200 mesh copper grids. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JOEL JEM-100CX/II electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV.

Cell internalization assays

Target monolayer cells were trypsinized to a single-cell suspension and plated on 24-well plates at 1 × 10<sup>6</sup> cells per well in RPMI 1640 medium containing 10% FCS. The next day, the target cells culture medium was removed and HOZOT cells at 5 × 10<sup>6</sup> cells per 0.5 ml culture medium containing 10 ng/ml IL-2 per well were added into each well. Cells were incubated for the indicated times at 37°C in 5% CO<sub>2</sub>, washed in PBS and detached, and adherent cells were collected and cytospon onto glass slides in a Cytospin 2 (SHANDON, Inc., Pittsburgh, PA, USA) at 700 rpm on high acceleration for 3 min. Cytospin samples were stained with Wright’s Solution (Wako Pure Chemical Industries Ltd., Osaka, Japan). HOZOT cells surrounded by target cells, at least 50%, were considered to be internalized. Percentages were calculated from a total of 200 cells in at least three independent experiments.

CellTracker analyses

Target monolayer cultures were stained with CellTracker Green (CMFDA, Invitrogen) and HOZOT cells were stained with CellTracker Red (CMTPX, Invitrogen) for 30–45 min at 37°C in the absence of serum. Labeled HOZOT and target cells were washed in PBS twice, target monolayer cells were trypsinized to a single cell suspension, and mixed 5:1 (HOZOT:target) in a poly-L-lysine-coated 8-well chamber slide system (Nalge Nunc International, Naperville, IL) at 1 × 10<sup>5</sup> cells per well in growth medium. At the indicated times, cells were washed in PBS, and fixed in 4% paraformaldehyde for 20 min at room temperature. Fixed cells were washed with PBS and covered with 50% glycerol in PBS. Cells were visualized by microscopy on a Carl Zeiss Axioplan with an LSM 510 laser scanning (confocal) system and
Immunofluorescent microscopy

For β-catenin staining, cocultures of HOZOT and target cells were washed in PBS and cytotospun onto glass slides in a Cytospin 2 (SHANDON, Inc., at 700 rpm on high acceleration for 3 min. Cytospins were fixed in 4% paraformaldehyde for 20 min at room temperature. Fixed samples were washed in PBS and blocked in 1% FCS in PBS for 10 min at room temperature. Primary antibody (anti-β-catenin, Transduction Laboratories) was incubated for 20 min in a humidified chamber at room temperature. The secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, Invitrogen) was incubated for 20 min in a humidified chamber at room temperature. Nuclei were stained with Hoechst 33258.

Detection of apoptosis and cytotoxic assays

For apoptotic cell staining, cocultures of HOZOT and MKN-45 cells were incubated at 37°C for 4 and 8 h. At the indicated time points, detached cells were collected and cytotospun onto glass slides. Apoptotic HOZOT cells within target cells were detected with an apoptosis detection kit (TUNEL method, ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit, S7101) (Chemicon International, Inc., Temecula, CA, USA) according to the manufacturer’s instructions. For anti-cleaved caspase-3 staining, nuclei of MKN-45 cells were pre-labeled with Hoechst 33342 (5 μg/ml in PBS) at 37°C for 20 min, and next stained with CellTracker Red for 30 min in the absence of serum. Hoechst 33342 and CellTracker double-labeled MKN-45 (red) cells and HOZOT cell nuclei were cocultured in 24-well culture plate (Corning, NY, USA) at 37°C for 4 and 8 h. At the indicated times, detached cells were collected and cytopsinned onto glass slides. Cytospin samples were fixed in 4% paraformaldehyde for 20 min at room temperature. Fixed samples were washed in PBS and blocked in 1% FCS in PBS for 10 min at room temperature. Primary antibody (anti-cleaved caspase-3, Calbiochem) was incubated for 20 min in a humidified chamber at room temperature. The secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen) was incubated for 20 min in a humidified chamber at room temperature. After staining, TUNEL or anti-cleaved caspase-3-positive HOZOT cells within MKN-45 cells were counted with a Nikon Optiphoto microscope or fluorescent microscope at ×400 magnification. For inhibition study, HOZOT cells were individually pre-treated with 25 or 50 μM of caspase-3 inhibitor (Calbiochem, La Jolla, CA, USA) at 37°C for 1 h. After incubation, HOZOT cells were washed in PBS and cocultured with MKN-45 cells at 37°C for 8 h. Cell viabilities of target MKN-45 cells were counted with Trypan blue (0.1%) at the indicated times. Methods of cytchalasin treatment of HOZOT cells were previously described in Results.

Statistical analysis

Data were statistically analyzed by one-way ANOVA following Tukey–Kramer multiple comparison tests.

Supplementary data

Supplementary data for this article are available online at http://jmcb.oxfordjournals.org.

Conflict of interest: none declared.

References

Cell-in-cell activity of HOZOT


