Multidrug resistance of non-adherent cancer cells

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Metastases are the cause of 90% of human cancer deaths. Cancer *in situ* can usually be effectively removed by surgery. Once cancer cells disseminate from the original site and start to circulate in blood, lymph, or other body fluids, the disease becomes almost incurable. Here we show that cancer cells in a non-adherent, 3-dimentional growth pattern are highly drug resistant compared to their adherent counterparts that grow in monolayer, attaching to the wall of tissue culture plates. The non-adherent cancer cells retain the adhering potential and can attach to an appropriate surface to reacquire adherent phenotype. Once the non-adherent cancer cells become attached, they regain drug response, similar to the original adherent cells. A significant increase in the expression of CD133, CD44, Nanog, survivin, and thymidylate synthase was observed in the non-adherent cancer cells compared to their adherent counterparts, which may underlie the mechanisms of multidrug resistance of the cells. Since the non-adherent cancer cells cultured *in vitro* resemble the circulating metastatic cells *in vivo* in that both cells exhibit suspended non-adherent phenotype, possess re-attaching potential, and are highly drug resistant, we suggest that circulating metastatic cells can attach to an appropriate surface to gain adherent phenotype and subsequently acquire drug sensitivity. We propose that devices coated with cell attachment materials or small particles of extracellular

matrix and collagen that mimic the structural framework of real human tissues to which cells can attach and grow may be able to stabilize the circulating metastatic cells. Once the metastatic cells undergo attachment and become adherent, they gain drug sensitivity and can be killed by anticancer drugs that are either administered to the blood or conjugated to the devices.

The cancer stem cells (CSCs) have been highlighted lately, due to the opinion that these CSCs are the culprits for therapeutic resistance, metastasis, and recurrence of the diseases, and therefore, might be selected as the target of treatment[1-4]. Of the many properties that the CSCs possess, two characteristics came to our attention, *i.e.*, non-adherent, 3-dimentional growth phenotype and high drug resistance. While working on the isolation and maintenance of CSCs, we found that anticancer drug resistance of the cancer cells was associated with their suspended, non-adherent growth pattern.

We isolated CD133 positive cells from human colon carcinoma cell lines CBS, Moser, HCT113, Fet, Caco-2, and SW480 cells using anti-CD133 antibody and Magnetic Cell Separation Rack. We also isolated CD44 positive cells from human breast carcinoma cell lines MDA-MB-231, MDA-MB-435, MCF-7, HCC1937, HCC1806, and Hs578T cells using anti-CD44 antibody and the same device. The isolated-cells were cultured in stem-cell culture medium (StemPro medium: DMEM/F-12/GlutaMax, StemPro Growth Supplement, 1.8% BSA, FGF, Nodal, Noggin, and 2-Mercaptoethanol) to retain the cells in an undifferentiated stemness status. The isolated cells growing in the StemPro medium exhibited a suspended, non-adherent, 3-dimentional spherical growth phenotype compared to their adherent counterparts in the regular medium (5% FBS/SMEM) that showed monolayer attachment to the wall of tissue culture plate. Morphology of human

colon carcinoma CBS cells and breast carcinoma MDA-MB-231 cells were shown as representatives (Fig 1a and 1b). Later on we accidently found that the adherent phenotype could be acquired to the cells by simply placing the parental cells in a basic serum-free medium (DMEM/F-12/GlutaMax + 1.8% BSA) (Fig 1a-iii and b-iii). Furthermore, we found that the cancer cells acquired similar non-adherent morphology when cultured in regular medium (5% FBS/SMEM) but in an Ultra Low Attachment Surface (ULAS) plate (polystyrene coated with neutral charged, hydrophilic hydrogel) (Fig 1a-iv and b-iv). All the non-adherent cells proliferated in a fast pace and were fully viable, determined by the Vi-CELL XR Cell Viability Analyzer (Beckman Coulter), which pre-stain the cells to detect the viable cells from the total cell counting. We have also tested normal human colon epithelial cell lines NCM460 and NCM356 cells, as well as normal human breast cell line MCF-10A cells for their suspended non-adherent growth pattern. None of the cells survived in a non-adherent growth condition (data not shown), indicating that the anchorage-independent, non-adherent phenotype is unique to cancer cells.

With further study, we found that the non-adherent cells—from either the isolated cells in StemPro medium, the non-adherent cells in the basic serum-free medium, or the non-adherent cells in the ULAS plates—were equally resistant to anticancer drugs. As it is shown in Fig 2, the non-adherent CBS cells were almost completely resistant to fluorouracil (5-FU)—the drug of choice for colon cancer—at a dose of as high as 300 μM, though it killed more than 80% of the adherent cells in monolayer attachment growth (Fig 2a). Similarly, the non-adherent MDA-MB-231 cells were highly resistant to paclitaxel—the drug of choice for breast cancer—at a dose of 8 μM, while it killed almost 90% of the adherent cells (Fig 2b). The non-adherent cells were also multidrug

resistant. As it is shown in Fig 2c, the non-adherent CBS cells were resistant to all the FOLFIRI (5-FU, leucovorin, and SN-38)—the regimen of choice for colon cancer, the mitomycin C (MMC), and the paclitaxel. Similar results were obtained with all of the aforementioned cells tested.

Although non-adherent cancer cells exhibit a suspended growth pattern, they have not lost attachment potential. Once the cells were placed to a regular medium (5% FBS/SMEM) in normal tissue culture plates (polystyrene with negatively charged, hydrophilic surface), the cells quickly reacquired attachment to the wall of the plates and grew in a monolayer attachment pattern similar to the adherent cells (Fig 1c). Moreover, the re-attached cancer cells re-gained response to anticancer drugs just as the adherent cells (Fig 3).

We don't know why the non-adherent cancer cells are highly resistant at this moment. We did see, however, that the non-adherent cancer cells expressed a high level of so-called "cancer stem cell markers" such as CD133 and CD44 compared to the adherent cells (Fig 4) [5-7]. Nanog, a transcription factor functioning in maintaining embryo stem cells in the undifferentiated state[8], survivin, an anti-apoptotic molecule[9,10], and thymidylate sythase (TS), a key enzyme involved in the *de novo* synthesis of DNA which circumvents the efficacy of 5-FU[11,12], are also significantly up-regulated in the non-adherent cells (Fig 4a). Although the results suggested that the non-adherent cancer cells possess some CSCs properties that might have been the underlying mechanism of the drug resistance of the cells, the relationship of the non-adherent cancer cells with CSCs are far from understood. CSCs are defined as a distinct subpopulation of cancer-initiating cells that constitute a small percentage of the tumour bulk. It is believed that in the

general cancer cell population, only CSCs possess the stem cell-like characteristics including undifferentiated status, drug resistance, tumorigenesity, expression of stem cell markers, self-renewal, and metastasis[1,2,13,14]. This CSC theory is, however, not unchallenged. Some studies have reported that all cells from tumours are equally tumorigenic[15-17]. It seems that our results support the latter by showing that the non-adherent cancer cells, prepared from either the isolated stem cell marker-expressing cells or the general cell population in suspended growth pattern, are equally expressing stem cell markers and highly drug resistant. Moreover, once the non-adherent cells underwent attachment, the up-regulated CD133 and CD44 quickly reversed to the levels of adherent cells (Fig 4b). These results may suggest that some of the stem cells' characteristics, or specifically the drug resistance of cancer cells, are more likely determined by the microenvironment-controlled cell growth patterns rather than by the *bone fide* cancer stem cells.

Several conclusions can be drawn from the results described above. (1) All the cancer cells tested can grow in an anchorage-independent, non-adherent manner. (2) Non-adherent cancer cells are highly resistant. (3) Non-adherent cancer cells still possess adherent potential and can quickly reacquire the adherent phenotype when an appropriate surface and a serum-containing medium are provided. (4) Re-attached cancer cells possess the same response to anticancer drugs that the original adherent cells do. It is noteworthy to point out that although we only showed data from two representative cell lines in the article, we have obtained similar results from all the aforementioned cell lines, indicating that the phenomena observed are not specific to individual cell lines but universally exist in colon and breast cancer cells, or possibly cancer cells from other

tissues that we have not yet tested. We propose that the properties that the non-adherent cancer cells possess *in vitro* may also apply to cancer cells *in vivo*. The non-adherent cancer cells resemble circulating metastatic cancer cells in that both cells survive in an anchorage-independent, non-adherent manner and both cells possess the potential of re-attachment when a favourable condition is introduced. Like the non-adherent cells that are more resistant than their adherent counterparts, the circulating metastatic cells are also resistant to anticancer drugs[18]. Turning metastatic cancer cells from a non-adherent to an adherent phenotype may, therefore, increase the sensitivity of the non-adherent metastatic cells to anticancer drugs, as what happened in the *in vitro* cell culture condition. We have known from this study that an appropriate attachment surface and a serum-containing medium are the two requirements for non-adherent cells to undergo attachment. In a real human body, the serum can be ignored since body fluids contain a similar serum as FBS. Therefore, introducing an appropriate surface is the key issue to fulfilling this purpose. In the condition of *in vitro* cell culture, cell attachment proteins bind well to the negatively charged, hydrophilic surface of the polystyrene wall of the tissue culture plates. In real human tissues, the structural framework formed by fibroblasts and their synthesized extracellular matrix and collagen provide surface for cells to attach and grow. If we introduce devices or micro-particles with a surface coated and conjugated with all the necessary attachment materials, we may be able to adsorb the circulating cancer cells to the attachment surface. As we have already known from the *in vitro* tests, adherent cancer cells are much more sensitive to anticancer drugs. Thus, a standard dose of anticancer drugs in the blood or conjugated on the attachment surface would kill the metastatic cancer cells. We assume that other cells in blood or other body

fluids would not attach to the attachment surface since blood cells are naturally anchorage-independent and do not possess the potential of adhesion.

The non-adherent cancer cell model may also be applied in high-throughput screening of agents targeting CSCs or resistant cancer cells. In principle, the application of automated screening technologies could facilitate the identification of agents that kill CSCs or resistant cancer cells. However, the screening depends on the ability to propagate stable, highly enriched populations of CSCs *in vitro*, which is not currently possible for the CSCs of solid tumours[4]. The non-adherent cancer cells, although not yet being fully determined for their CSCs properties, do possess CSC characteristics of high drug resistance; the cells are also easy to prepare and maintain. Therefore, the non-adherent cancer cell model may well apply for such purposes.

METHODS

Cell culture. Human colon cancer cell lines CBS, Moser, HCT113, Fet, Caco-2, and SW480 cells and human breast cancer cell lines MDA-MB-231, MDA-MB-435, MCF-7, HCC1937, HCC1806, and Hs578T cells were maintained in SMEM medium (MEM medium supplemented with sodium bicarbonate, peptone, vitamins, amino acids, and 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO). CD133(+) colon carcinoma cells and CD44(+) breast carcinoma cells were maintained in StemPro medium (1×DMEM/F-12/GlutaMax, 1×StemPro Growth Supplement, 1.8% BSA, 8 ng/ml FGF, 10 ng/ml Nodal, 10 ng/ml Noggin, and 0.1 mM 2-Mercaptoethanol) (Invitrogen Co.). Preparation of the non-adherent cancer cells in the basic serum-free medium: the cancer cells were detached using 0.25% trypsin-EDTA solution and washed with cold 1×PBS buffer and

then placed and maintained in the basic serum-free medium (1×DMEM/F-12/GlutaMax + 1.8% BSA). Preparation of the non-adherent cancer cells in ULAS flask: the cancer cells were detached using 0.25% trypsin-EDTA solution and washed with cold 1×PBS buffer and then placed and maintained in SMEM medium in ULAS flasks (polystyrene coated with neutral charged, hydrophilic hydrogel) (Corning Inc.)

Magnetic cell separation. CD133(+) colon cancer cells were labeled with anti-CD133 antibody (Miltenyi Biotec) and CD44(+) breast cancer cells were labeled with anti-CD44 antibody (Cell Signaling), followed by incubation with magnetic-beads conjugated goat anti-mouse secondary antibody (New England BioLab) and separation of the labeled cells from the unlabeled cell population using a Magnetic Separation Pack (New England BioLab).

Morphology. OLYMPUS CKX41SF microscope connected with OLYMPUS DP-12 camera (Olympus Co., Japan) were used to analyze and record the morphology of the cells.

Cell viability assays. A Vi-Cell^{TM} XR Cell Viability Analyzer (Beckman Coulter, Inc., Fullerton, CA) was used to determine the number of viable cells in a suspension after exposure to the anticancer drugs indicated in the figures.

Western analysis. Standard Western blot procedures were performed.

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END NOTES

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FIGURE LEGENDS

Figure 1. Morphological characteristics of the non-adherent and the adherent cancer cells. a, CBS parental cells in SMEM medium (i); CBS CD133(+) cells in StemPro medium (ii); CBS non-adherent cells in basic serum-free medium (iii); and CBS non-adherent cells in SMEM medium in an ULAS plate. **b,** MDA-MB-231 parental cells in SMEM medium (i); MDA-MB-231 CD44(+) cells in StemPro medium; MDA-MB-231 non-adherent cells in basic serum-free medium (iii); and MDA-MB-231 non-adherent cells in SMEM medium in an ULAS plate. **c,** CBS CD133(+) in StemPro medium (i); CBS CD133(+) cells were replaced in SMEM medium for 12 h (ii), 24 h (iii), and 48 h (iv).

Figure 2. Response of the non-adherent and the adherent cancer cells to anticancer drugs. a, CBS cells in the different culture conditions indicated were exposed to 5-FU at the doses indicated for 24 h. **b,** MDA-MB-231 cells in the different culture conditions indicated were exposed to paclitaxel at the doses indicated for 24 h. **c,** CBS cells in the different culture conditions indicated were exposed to FOLFIRI (90 μM 5-FU, 28 μM leucovorin, and 40 nM SN-38), MMC, and paclitaxel for 24 h. The viable cells were determined as described in the Methods. Results are expressed as mean ± s.e.m. of triplicate experiments.

Figure 3. Response of the re-attached cancer cells to 5-FU. a, the non-adherent CBS CD133(+) cells were re-placed in SMEM medium to get the cells attached to the wall of the tissue culture plate. **b,** the non-adherent CBS cells in the basic serum-free medium were re-placed in SMEM medium to get the cells attached to the wall of the tissue culture plate. **c,** the non-adherent CBS cells in an ULAS plate were re-placed in a normal tissue

culture plate to get the cells attached to the wall of the tissue culture plate. The re-attached cancer cells and the parental cells were exposed to 5-FU for 24 h and the viable cells were determined as described in the Methods. Results are expressed as mean ± s.e.m. of triplicate experiments.

Figure 4. Up-regulation of several molecules in the non-adherent cancer cells. **a,** lane 1, CBS parental cells in SMEM medium; lane 2, CBS CD133(+) cells in StemPro medium; lane 3, CBS non-adgerent cells in basic serum-free medium; lane 4, CBS non-adherent cells in SMEM medium in an ULAS plate. The expression of the proteins indicated was determined by Western analysis. **b,** lane 1, CBS parental cells in SMEM medium; lane 2, CBS non-adherent cells in a ULAS plate; lane 3, CBS non-adherent cells that were re-placed in a normal tissue culture plate for 24 h; lane 4, CBS non-adherent cells that were re-placed in a normal tissue culture plate for 48 h; lane 5, CBS non-adherent cells that were re-placed in a normal tissue culture plate for 72 h. The expression of the proteins indicated was determined by Western analysis. β-actin expression was used as internal controls for equal protein loading.