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Simultaneous Evaluation of Cellular Vitality and Drug Penetration in Multicellular Layers of Human Cancer Cells

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ABSTRACT – The multicellular layers (MCL) of human cancer cells is a three dimensional (3D) *in vitro* model for human solid tumors which has been used primarily for the assessment of avascular penetration of anti-cancer drugs. For anti-cancer drugs with penetration problem, MCL represents a good experimental model that can provide clinically relevant data. Calcein-AM is a fluorescent dye that demonstrates the cellular vitality in a graded manner in cancer cell culture system. In the present study, we evaluated the use of calcein-AM for determination of anti-proliferative activity of anti-cancer agents in MCL model of DLD-1 human colorectal cancer cells. Optical sectioning of confocal imaging was compromised with photonic attenuation and penetration barrier in the deep layers of MCL. By contrast, fluorescent measurement on the cryo-sections provided a feasible alternative. Cold pre-incubation did not enhance the calcein-AM distribution to a significant degree in MCL of DLD-1 cells. However, the simultaneous determination of drug penetration and cellular vitality appeared to be possible in drug treated MCL. In conclusion, these data suggest that calcein-AM can be used for the simultaneous determination of drug-induced anti-proliferative effect and drug penetration in MCL model.

Key words – Multicellular layers (MCL), Solid tumor, Penetration, Calcein-AM, Confocal imaging

The monolayer culture model serves as a suitable tool for mechanistic and biochemical studies with human cancer cells. However, it lacks the clinical relevance to human solid tumors. Although national cancer institute cell line panel (NCI-60 cell line) test has been used for anti-cancer drug screening, it presents limitations due to oversimplified two dimensional culture model.[1] Three dimensional (3D) culture of human cancer cells is a well established model for effective anti-solid tumor drug screening. The 3D culture model simulates many of the *in vivo* conditions of solid tumors. Lack of vasculature, abundance of extracellular matrix (ECM), and cell-cell communication (collectively Multi-cellular resistance) are *in vivo* like conditions which are maintained in 3D culture system.[2-4] Different types of 3D culture models have been developed for the anti-cancer drug screening.[5,6] Multicellular layers (MCL) is a 3D model for extravascular diffusion assessment of chemotherapeutics in solid tumors, which has been introduced by Wilson and coworkers, and used for pharmacokinetic studies of anti-cancer agents.[7,8] In contrast to other 3D models, MCL model has been successfully used for mathematical modeling of pharmacokinetic-pharmacodynamic (PK-PD) studies of chemotherapeutic agents.[9]

Calcein-AM is a fluorescent dye that can penetrate into cells, transformed into fluorescent form and trapped only in vital cells. The fluorescence intensity is proportional to the number of cells and to the proliferative activity of the cells. The optical sectioning using a confocal microscope has been reported to assess vitality in the deep layers of multicellular spheroids stained with calcein-AM.[10,11]

Drug penetration has been recognized as one of the major determinants in the treatment of human solid tumors.[12] Paclitaxel and doxorubicin are very important chemotherapeutic agents in the treatment of various human solid tumors. Unfortunately, both paclitaxel due to its microtubule binding and high molecular weight and doxorubicin due to its high DNA binding and endosomal sequestration suffer from slow penetration into human solid tumors.[13,14] MCL model made it possible to study the drug penetration kinetics without estimating complex pharmacokinetic (PK) parameters in the *in vivo* xenograft model. Especially for high molecular weight drugs with high protein binding or intracellular sequestration, improved tissue penetration is considered to provide better drug efficacy.

In the present study, we evaluated the feasibility of PK-PD studies in MCL model using calcein-AM and fluorescent drugs. Problems in optical sectioning with confocal microscope are presented and alternative methodology using cryo-section of MCL is suggested.

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Materials and Methods

Chemicals and reagents

Paclitaxel-rhodamine (PTX-Rd) was a kind gift from Dr. S.Y. Kim (Han-yang University, Seoul, Korea). Calcein-AM was purchased from Molecular probes, Inc. (Eugene, OR). Cell culture reagents and transwell insert® (0.4 μm microporous membrane) were purchased from Gibco BRL (Rockville, MD) and Corning Costar (Acton, MA) respectively. Doxorubicin (DOX), and other reagents unless otherwise noted, were purchased from Sigma Chemical (St. Louis, MO).

Cell culture condition

The human colorectal cancer cell line, DLD-1 was obtained from Korea cell line bank (Seoul, Korea). Cells were maintained in RPMI-1640 supplemented with 100 μg/mL streptomycin, and 100 unit/mL penicillin and 10% heat-inactivated fetal bovine serum in a humidified 5% (v/v) CO_2 atmosphere at 37 °C.

Culture of multicellular layers

DLD-1 cells were grown on collagen-coated microporous (0.4 μm) membranes in transwell inserts at a plating density of 3×10^5 cells/100 μL. The six inserts were placed in a culture jar supplemented with 150 mL RPMI-1640 media with continuous stirring in the bottom chamber as previously described.[7,15] At the end of growing period, transwell inserts containing MCL were transferred to six-well plate supplemented with 7 mL of media in the bottom chamber. For the whole period of culture (five days) in the jar and subsequent culture in six-well plate, cells were maintained in an incubator supplied with 5% CO_2 and humidified air at 37 °C.

Determination of calcein-AM signal in monolayer culture of cells

Exponentially growing cells were harvested using 0.25% trypsin-EDTA and seeded in 96-well plates at a seeding density of 1×10^4 to 2.5×10^5 cells/well. Plates were incubated overnight and then exposed calcein-AM containing media (2 μM and 4 μM) for 45 min. After the incubation, cells were washed twice with ice cold PBS, and fluorescence intensity was measured at $\lambda_{Ex/Em}$=494/517 nm.[16]

Confocal imaging for the calcein-AM stained MCL and stack of cells

The vitality in the deep structure of MCL was determined using calcein-AM vitality probe. Briefly, calcein-AM was applied in the top chamber of the MCL at a final concentration of 40 μM, and incubated for 45 min at 4 °C followed by 15 min in room temperature (RT) or incubated at RT for 60 min. Immediately after the incubation, the MCL's were cut off the transwell insert, inverted on a cover slip and examined with confocal laser scanning microscope (MRC 1024 MP, Bio-Rad, CA). Optical sections were acquired at 10 μm increments along the z-axis of the sample. For the stack of cells, 2×10^7 cells were incubated with 40 μM of calcein-AM for 45 min at RT, and then stacked on a cover slip by centrifugation at 2000 rpm for 20 min. Stacked cells were also subjected to confocal laser scanning microscope for 10 μm optical sections. Optical sections of the light field were used to define the cell surface and transwell membrane or glass surface coordinates which were taken as the start and stop signal, respectively.[10] Fluorescence intensity was measured at $\lambda_{Ex/Em}$=488/517 nm. Fluorescent images were analyzed by using Optimas ver. 6.5 and plotted against the depth along the vertical axis. Six measurements were taken for six different locations per each sample.

Determination of the vitality and drug distribution in MCL using cryo-sections

Calcein-AM staining was performed as described above. For simultaneous determination of calcein-AM and drug penetration, MCL's were exposed to PTX (100 μM) or DOX (100 μM) for 72 hr, and then calcein-AM for 1 hr. MCL's were embedded in OCT compound, and snap frozen in liquid nitrogen. The MCL's were cut off the transwell and frozen sections (20 μm) were obtained (Cryostat microtome, CM 1800, Leica, Germany), and examined with fluorescent microscope (Olympus, AX70, TR-6A02, Tokyo, Japan). Fluorescence intensity was measured at $\lambda_{Ex/Em}$=488/517 nm for calcein-AM and at $\lambda_{Ex/Em}$=482/505 nm for DOX and PTX-Rd. Images were analyzed using Optimas ver. 6.5 and line morphometric curves of the fluorescent intensities were plotted.

Results

Calcein-AM signal in DLD-1 cells grown as monolayer

DLD-1 was exposed to two different concentration of calcein-AM at various cell densities. The fluorescent signal intensity increased at higher dye concentration. The intensity also increased with cell number, however the increase was linear till the cell seeding density of 1.5×10^5 cells. At higher density, a plateau fluorescence signal was achieved. It was noticed that incubation at 4 μM of calcein-AM showed less than 2 fold increase in intensity compared to 2 μM (Figure 1).

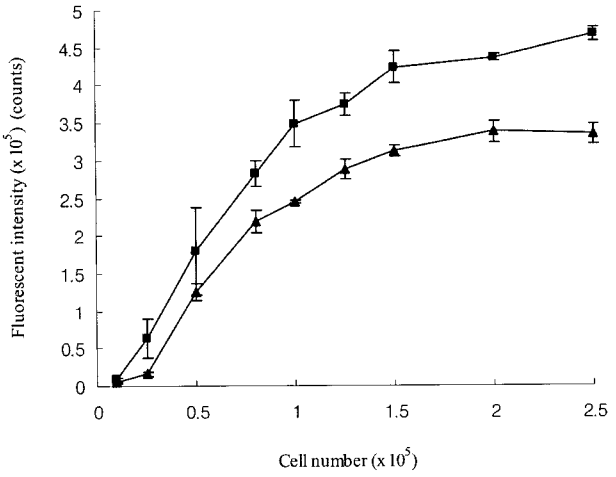

Figure 1–Relationship between cell number and fluorescence intensity after exposure to 2 μM (▲) or 4 μM (■) of calcein-AM for 45 min at RT. Data are expressed as mean±S.E. (n=3).

Calcein-AM distribution in the deep layers of the MCL and stack of DLD-1 cells

The optical sections taken along the z-axis through the multicellular layers of tumor cells showed fluorescent signal intensities in a parabolic profile (Figure 2C). Mean field intensity increased with depth and reached to the maximum after about 2 optical sections (20 μm from the surface point). Rapid decrease in fluorescence intensity was observed after maximum intensity. The decrease was more prominent in MCL compared to the stacked cells and no fluorescence signal could be detected in a depth further than 80 μm in MCL. Decrease in fluorescence intensity was less in the stacked cells and fluorescence was detected through the whole thickness of the stacked cells (Figure 2C). Fluorescence was detected through the whole thickness of the stacked cells (up to 140 μm depth) (Figure 2B). These data indicate that calcein-AM penetration is a technical problem in assessing the vitality in MCL of tumor cells.

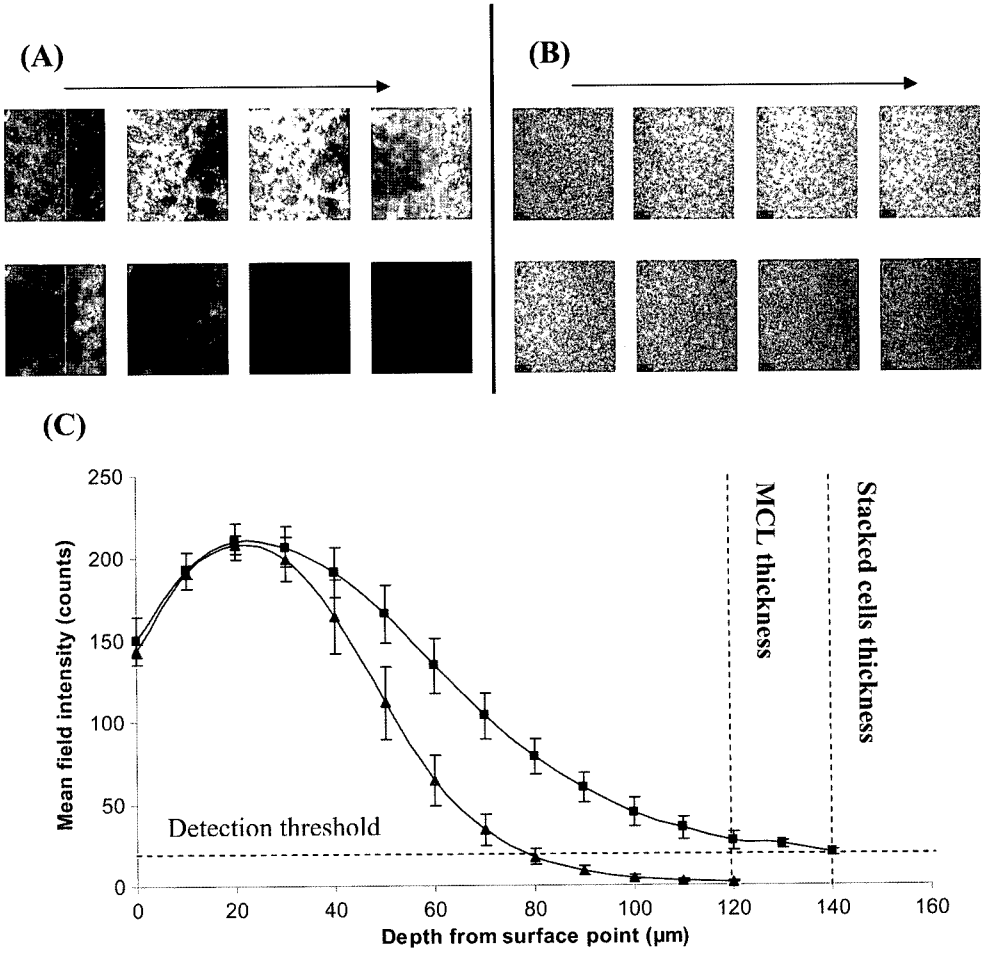

Figure 2–Three dimensional distribution of calcein-AM in DLD-1 formed as MCL (A) or stacked cells (B) after incubation with calcein-AM (40 μM) for 45 min at RT. Images were produced by optical sectioning from the top layer till the bottom layer at 10 μm increments along z-axis of the sample. Fluorescence intensities of the optical sections of MCL (▲) and stacked cells (■) along the z-axis (C). Data are expressed as mean±S.E. (n=6).

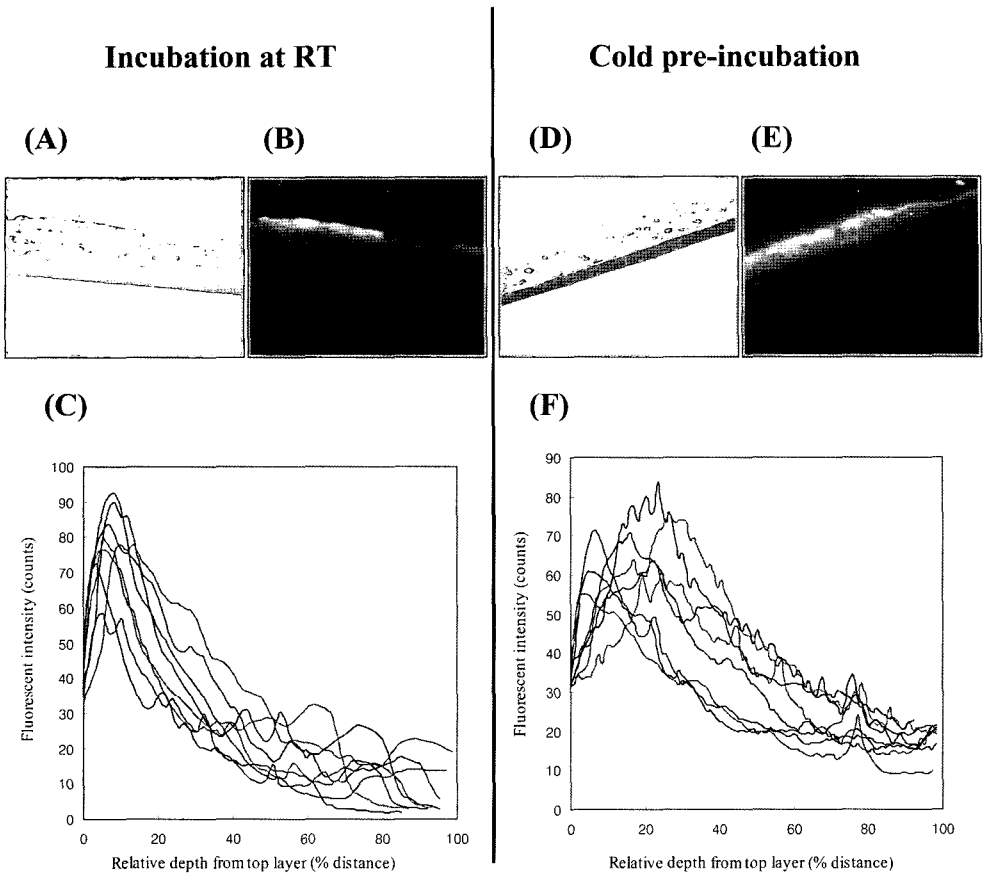

Figure 3–Distribution of calcein-AM in MCL's of DLD-1 cell line after exposure to calcein-AM (40 μM) for 60 min at RT (A, B and C) or after 45 min at 4 °C followed by 15 min at RT (D, E and F). MCL's were snap-frozen immediately after exposure. Phase contrast images (A and D) and fluorescence images (B and E) are shown. Profile of fluorescence intensities are obtained from several representative morphometric lines across the MCL frozen sections (C and F).

Penetration of calcein-AM through the layers of MCL

The frozen sections of DLD-1 MCL confirmed that calcein-AM did not penetrate more than 30-40% of the thickness of the MCL after treatment with calcein-AM at RT compared to 50-60% of the thickness after cold pre-incubation (Figure 3A and B). The line morphometric analysis shows high accumulation of the calcein-AM in the top layers (Figure 3C). The cold pre-incubation enhanced the distribution of calcein-AM to some extent. (Figure 3D and E). The line morphometric analysis showed broader peaks in cold pre-incubated samples compared to sharp peaks for the RT samples. The difference between maximum and minimum fluorescence signal is greater in samples treated in RT compared to samples treated after cold pre-incubation. The cold pre-incubation diminished the degree of signal fluctuation compared to that at the RT sample (Figure 3C and F).

Simultaneous determination of the vitality and the distribution of chemotherapeutic agents within the MCL

In order to evaluate the feasibility of simultaneous staining of fluorescent vital dye and fluorescent drugs, calcein-AM images were obtained in MCL exposed to PTX-Rd or DOX for 72 hr. After 72 hr exposure, PTX-Rd and Dox showed full penetration through the MCL. DOX was found to be localized to the nuclear compartment while PTX-Rd was distributed to all cellular and extracellular compartments. DOX was homogenously distributed throughout the whole thickness of the MCL while PTX-Rd showed higher accumulation in the top proliferating layers. In addition, calcein-AM showed full penetration which may be attributed to drug-induced changes in tissue architecture, compared to non-drug treated MCL's (Figure 4).

Discussion

Wilson and coworkers introduced the MCL as one of the 3D culture model which has been used primarily for PK studies such as drug penetration and metabolism.[15,17,18] In addition to PK studies, MCL is used for pharmacodynamic studies in con-

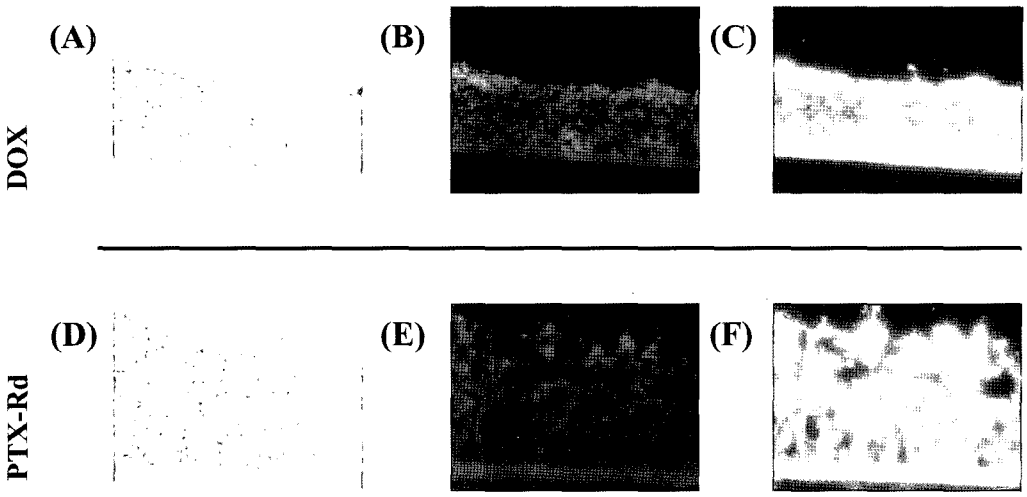

Figure 4–Simultaneous determination of calcein-AM and drug distribution in the frozen sections of MCL. MCL's were treated with 100 μM of PTX-Rd or 100 μM of DOX for 72 hr followed by 1 hr incubation with calcein-AM (40 μM). Phase contrast images for the MCL's are shown in A and D. Distribution of drug and calcein-AM is shown in the same section for DOX (B and C) and PTX-Rd (E and F), respectively.

junction with cytotoxicity assays including conventional SRB chemosensitivity assay, DNA flow cytometry, clonogenic assay and other assays.[9,19] These cytotoxicity assays disrupt the 3D architecture, resulting in average cytotoxicity data output. While MCL model has been used for PK-PD studies for anti-cancer agents using mathematical modeling, our goal was to assess PK-PD relationship in a more direct way eliminating the complex mathematical modeling.[9]

Calcein-AM coupled with the confocal imaging technique is suggested as the best alternative to provide a suitable method for vitality evaluation in the 3D model.[10] The vitality signal induced by calcein-AM was shown to be cell number- and concentration-dependent (Figure 1). At a certain cell number (1.5×10^5 cells), a plateau of fluorescent intensity was observed regardless of the calcein-AM concentration applied. These results can be attributed to the cell count/unit area at which the plateau was noticed (5×10^3 cells/mm^2). This cell count per unit area is expected to be similar to the cell density at each horizontal plane of the MCL of DLD-1 cell line in our experiment. This suggests that higher concentration of calcein-AM than 4 μM might be necessary in order to avoid expected substrate depletion. Based on this data higher concentration (40 μM) was used and found enough to obtain fluorescence intensity through out the layers of MCL (Figure 4C and F). This high concentration is similar to that used in the literature: 40 μM was used in spheroid culture system.[10]

As shown in the fluorescent intensity distribution profile of stacked cells, calcein-AM fluorescent signal suffers from photonic attenuation (Figure 2B), which was reported previously. Wartenberg and coworkers suggested a mathematical model for photonic attenuation and used it for signal correction.[10] However, our data indicated that the photonic attenuation is complicated by a penetration problem. P-glycoprotein (P-gp) was identified as the primary reason for the poor distribution of fluorescent compounds in solid tumor models; Tannock and coworkers suggested P-gp as a penetration enhancer in solid tumors.[17,20,21] Cold pre-incubation was recommended to improve calcein-AM distribution in multicellular spheroids of U343 cell line to a sufficient degree. Nonetheless, our data obtained in MCL of DLD-1 cell line did not confirm this recommendation.[10] Reducing the efflux mechanism did not improve the vitality probe distribution through the bottom layers of MCL in the absence of other treatments (Figure 3).

3D distribution of DOX could be determined in multicellular cancer spheroids by using confocal laser scanning microscopy.[11] In addition, the penetration of PTX could be determined using the compound labeled fluorescent moiety (BODIPY) or rhodamine as demonstrated in our study.[21] We showed the feasibility of simultaneous determination of the penetration of an anti-cancer agent together with the vitality probe distribution.

Conclusion

In the current work, we provide supportive data for the potential use of calcein-AM as a vitality probe in drug-treated MCL. In addition, we demonstrated that the penetration of DOX or PTX can be simultaneously determined in the cryo-

sections of MCL. These data suggest that calcein-AM can be used for the simultaneous determination of drug-induced anti-proliferative effect and drug penetration in MCL model.

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