A Microplate Assay for Selective Measurement of Growth of Epithelial Tumor Cells in Direct Coculture with Stromal Cells

MANABU KAWADA, YUYA YOSHIMOTO, KAZUHISA MINAMIGUCHI, HIROYUKI KUMAGAI, TETSUYA SOMENO, TOHRU MASUDA, MASAaki ISHIZUKA and DAISHIRO IKEDA

Drug Development Unit, Numazu Bio-Medical Research Institute, Microbial Chemistry Research Center, Numazu-shi, Shizuoka 410-0301, Japan

Abstract. Stromal cells play an important role in regulating epithelial malignancies through diffusible factors and adhesion. Modulation of the tumor-stromal cell interaction is an attractive target for new antitumor strategies. To screen for a modulator of the interaction, we have now developed a quantitative colorimetric assay for measurement of tumor cell growth in coculture with stromal cells using rhodanile blue dye. Rhodanile blue specifically stained cytokeratin-positive tumor cells in the coculture. When human prostate carcinoma cells LNCaP, PC-3 and DU-145 were cocultured with normal prostate stromal cells (PrSC) in a microplate, growth of the prostate cancer cells in the coculture was selectively measured by the rhodanile blue staining method. Using this system, we searched for a modulator of the tumor-stromal cell interaction among clinically used drugs and natural products. As a result, we found that 5-fluorouracil, bleomycin and phthoxazolin A inhibit prostate cancer cell growth more strongly in coculture with PrSC than that in monoculture. Without need to pre-label cells and transfect a marker gene, our new method is simple, rapid and thus useful for screening for modulators of the tumor-stromal cell interaction. Furthermore, our results suggest that low molecular weight compounds modulate the tumor-stromal cell interaction.

Solid tumor tissue is composed of tumor cells and surrounding stroma including extracellular matrix, fibroblasts, macrophages and endothelial cells. In fact, histological analyses show that tumor tissues apparently contain stromal cells (1, 2). Growth of tumor cells as well as epithelial cells in normal tissues is regulated by the stromal cells through diffusible factors and adhesion (3, 4). Thus, tumor-stromal cell interactions significantly participate in tumor growth, invasion and metastasis. It has been reported that these interactions may contribute to the development of some types of tumor such as breast and prostate cancer (5, 6).

Many researchers have investigated the effect of fibroblasts, as a major cellular component of the stroma, on epithelial tumor cell growth. Several data reveal that fibroblasts accelerate the growth of tumor cells such as breast and prostate cancer in vitro and in vivo (7, 8). However, there are specificities between tumor cells and fibroblasts. The growth of prostate and breast cancer cells is stimulated by fibroblasts derived from the respective organ, but not by fibroblasts of other origin (9-12). By contrast, there is evidence that fibroblasts can also suppress the growth of tumor cells (13, 14). Dermal fibroblasts suppress growth of the early stage melanoma (15) and fibroblasts derived from normal mammary tissues suppress the growth of breast tumor cells (16).

Thus, stromal cells such as fibroblasts may promote or suppress tumor growth in vitro. Therefore, it may be possible to control tumor growth by modulating the tumor-stromal cell interaction. To study whether stimulation of stromal cells by external factors results in modulation of tumor cell growth, we used prostate cancer cells as a model. We have previously reported that the growth of LNCaP human prostate carcinoma cells is significantly inhibited by coculture with normal fibroblasts in the presence of IL-1β or TNF-α through secretion of IL-6 from the normal fibroblasts (17). This result suggested that modulation of the tumor-stromal cell interaction could be a target for cancer chemotherapy.

We also applied the coculture method for screening new anticancer agents. Such a screening method must be rapid and easily handled. However, it is difficult to selectively measure the growth of only one cell type in the coculture system. Several methods such as inserted chambers, pre-labeling cells with a fluorescent dye and transfection of a
marker gene are used for coculture experiments (16, 18-20) but, with respect to cost and quantitative measurement of cell growth, they are not suitable for screening of many samples. Rhodanile blue dye, a mixture of Rhodamine B and Nile blue, has been reported to preferentially stain epithelial cells (21). We developed a new screening method for coculture using rhodanile blue dye.

In this report we describe a quantitative colorimetric assay method for selective measurement of the growth of epithelial tumor cells in coculture using a microplate. Furthermore, our results suggest that low molecular weight compounds modulate the tumor-stromal cell interaction.

**Materials and Methods**

Reagents. Rhodanile blue was purchased from Aldrich (Milwaukee, WI, USA). Insulin, hydrocortisone and anti-pan-cytokeratin (clone C-11) were obtained from Sigma (St. Louis, MO, USA). Transferin was from Wako Pure Chemical Industries (Tokyo, Japan). Recombinant human basic-FGF was from Pepro Tech (London, England). Alexa Flour® 488 anti-mouse IgG was from Molecular Probes (Eugene, OR, USA).

Cells. Androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cells were obtained from Dainippon Seiyaku (Osaka, Japan). Androgen-independent DU-145 human prostate cancer cells were from the American Type Culture Collection. Human normal prostate stromal cells (PrSC) were from BioWhittaker (Walkersville, MD, USA). Prostate cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 100 units/ml penicillin G and 100 µg/ml streptomycin at 37°C with 5% CO2. PrSC was maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin G, 100 µg/ml streptomycin, ITH (5 µg/ml insulin, 5 µg/ml transferrin and 1.4 µM hydrocortisone) and 5 ng/ml basic-FGF at 37°C with 5% CO2.

**Coculture experiment.** PrSC was first inoculated in a 96-well plate at 5000 cells/well in 100 µl of DMEM with the indicated supplements. Test samples were added into the well and the cells were cultured to confluence for 2 days. Then 10 µl of prostate cancer cell suspension (5000 cells) in serum-free DMEM was inoculated onto a monolayer of PrSC and the cells were further cultured for 3 days. For moniculture of prostate cancer cells, first only assay medium with test samples was inoculated for 2 days at 37°C, then prostate cancer cells were inoculated as described above and further cultured for 3 days.

**Rhodanile blue staining.** Cultured cells were fixed for 15 min by adding 50 µl of 5% glutaraldehyde in phosphate-buffered saline (PBS) directly into a 96-well plate. After washing three times with tap water, the plate was dried. Then the cells were stained for 15 min by adding 50 µl of 0.2% rhodanile blue dye in distilled water. After washing seven times with tap water and drying, the dye was eluted with 100 µl of 50% ethanol and absorbance at 550 nm was measured using a microplate reader, Labsystems Multiskan MS (Dainippon Seiyaku, Osaka, Japan). The absorbance at 550 nm of the medium alone and the PrSC alone was subtracted from the values in monoculture and coculture, respectively, as a background value. The absorption spectrum of the eluted dye was analyzed by a spectrometer 228A (Hitachi, Tokyo, Japan). The MTT assay was performed as previously described (17).

**Immunofluorescence.** The cells stained with rhodanile blue dye were washed three times with PBS and then blocked with 10% FBS in PBS. After washing with PBS, the cells were incubated with anti-cytokeratin antibody in 1.5% FBS in PBS and then with Alexa Flour® 488 anti-mouse IgG. The cells were examined using a fluorescence microscope, LEICA DM IRB (Wetzlar, Germany).

**Results**

**Rhodanile blue staining for coculture.** We have designed a coculture system of human prostate cancer cells and normal human prostate stromal cells (PrSC). To reflect direct cell-cell contact in coculture, we first inoculated PrSC and then cocultured prostate cancer cells on a monolayer of PrSC. In order to selectively measure growth of the prostate cancer cells in coculture, we used rhodanile blue dye, a mixture of Rhodamine B and Nile blue, which has been reported to preferentially stain epithelial cells (21), for staining the tumor cells. As shown in Figure 1, rhodanile blue selectively stained LNCaP prostate cancer cells, but not PrSC even in the coculture of both cell types. To confirm that rhodanile blue dye specifically stains tumor cells, rhodanile blue-stained cells were further applied to immunofluorescence with anti-cytokeratin, a specific marker of epithelial cells. As a result, cytokeratin-positive cells were identical with the rhodanile blue-stained cells (Figure 1D). Rhodanile blue also selectively stained other cancer cell lines including DU-145 and PC-3 prostate cancer cells (data not shown). To use rhodanile blue staining (RBS) for the evaluation of cancer cell growth, we had tried several procedures and found it best if the cells were fixed by glutaraldehyde and the dye was eluted by 50% ethanol as described in Materials and Methods. The absorption spectrum of the eluted dye showed that the absorbance at 550nm was high for prostate cancer cells and minimal for PrSC (Figure 2A). Thus, we evaluated the cancer cell growth in coculture measuring absorbance at 550nm of rhodanile blue-stained cells.

The RBS method showed that the absorbance at 550nm correlated well with the numbers of prostate cancer cells in coculture with PrSC as well as in monoculture (Figure 2B). Furthermore, compared with the MTT method, in which values of MTT formazan included both cancer cells and PrSC in coculture (17), the RBS method facilitated measurement of the growth of cancer cells in coculture more clearly than the MTT method (Figure 2C). As described below, the growth of LNCaP cells was promoted by coculture with PrSC cells and the promoting effects were better detected by RBS than by MTT (Figure 2C). Thus, the RBS method is suitable for the coculture system.
Optimization of coculture system. Stromal cells may stimulate or suppress tumor cell growth in vitro. To optimize the coculture system for screening, we first examined the effect of serum concentrations with or without ITH (insulin, transferrin and hydrocortisone). When LNCaP cells were cocultured with PrSC cells with various concentrations of FBS, the growth of LNCaP cells was stimulated at concentrations higher than 0.1% FBS compared with the growth in monoculture, but the growth-promoting effect was only partial. Some medium additives such as insulin and transferrin are often used in growth factors-deficient media (21, 22). In the presence of ITH the growth of LNCaP cells in monoculture was almost the same as that in the absence of ITH (Figure 3A and B). By contrast, the growth of LNCaP cells in coculture with PrSC cells in the presence of ITH was significantly stimulated at concentrations of 0.1 and 1% FBS (Figure 3A and B). Since PrSC grew well in the presence of ITH (data not shown), we used ITH for further experiments. Coculture of the other prostate cancer cell lines with PrSC cells in the presence of ITH revealed that the growth of DU-145 cells was also promoted by coculture with PrSC, but the growth of PC-3 cells was not affected (Figure 3C and D). Although all the prostate cancer cell lines tested here did not respond to coculture with PrSC cells, the growth of LNCaP and DU-145 cell lines were found to be promoted by PrSC and the growth-promoting effect was significant at lower concentrations of serum in the presence of ITH.

Effect of various drugs on coculture. We examined the effect of various antitumor drugs on the coculture of LNCaP with PrSC cells in the presence of ITH and 0.1% FBS. As shown in Figure 4, almost all antitumor drugs inhibited LNCaP cell growth in both cultures to a similar extent. All drugs except mitomycin C at the tested high concentrations showed no cytotoxicity against PrSC cells. However, among the drugs tested, only 5-fluourouracil (5-FU) and bleomycin (BLM) inhibited the growth of LNCaP cells in coculture with PrSC more strongly than that in monoculture (Figure 4). When we examined the effect of both drugs on the other cell lines, there were differences in their actions. 5-FU significantly inhibited the growth of LNCaP and DU-145 cell lines in the coculture, but BLM only the growth of LNCaP cells (Figure 5).

We also tested certain microbial metabolites for their possible effect on tumor-stromal cell interactions. A microbial cultured broth was found to inhibit the growth of LNCaP in coculture with PrSC cells more strongly than that in monoculture without any cytotoxicity against PrSC. The active material was purified and identified as phthoxazolin A (MW 290), which was first reported as an inhibitor of cellulose biosynthesis (23-25). Phthoxazolin A inhibited the growth of all the tested prostate cancer cell lines in coculture with PrSC more strongly than that in monoculture (Figure 5).

Discussion

Tumor growth in vivo necessarily requires angiogenesis. It has been shown that stromal fibroblasts induce angiogenesis in a three-dimensional coculture system of tumor cells, epithelial cells and fibroblasts (26). Stromal cells such as fibroblasts not only modulate tumor cell growth but also play an important role in angiogenesis. Furthermore, genetic alterations occur in tumor-adjacent stroma in vivo as well as in tumor cells suggesting the heterogeneity of stroma in tumor tissues (27). Thus, modulation of the tumor-stromal cell interactions is an attractive target for new antitumor strategies. Several data reveal that there are organ-specific interactions between tumor cells and stroma derived from the tumor-originated tissues (9-12). Moreover, tumor cells secrete stroma-inducing factors such as VEGF, TNF-α and PDGF preferentially towards a basolateral stroma-oriented direction (28). Therefore, we designed a coculture system of prostate carcinoma cells and PrSC cells by inoculating tumor cells on a lawn of stromal cells.

We have described a novel in vitro assay method using rhodanine blue dye for the study of a coculture system that can be used to screen for modulators of tumor-stromal cell interactions. Several researchers have previously examined tumor-stromal cell interactions in coculture, some of them utilizing the inserted chambers in which a microporous membrane separated two cell types in coculture (16, 18, 19). However, the cost of the inserted chamber assay make it unsuitable for screening of many samples. Furthermore, in that system the influence of direct cell-cell contact between stromal cells and tumor cells was excluded. In addition to secreted factors, the direct cell-cell contact greatly influenced the properties of the coculture. Olumi et al. presented a new assay method for coculture in which two cell types were directly in contact (20). They labeled fibroblasts with a fluorescent dye and analyzed the growth of tumor cells in coculture with the fibroblasts by flow cytometry. However, this method, again, can not handle many samples for screening. By contrast, rhodanine blue distinguishes the two cell types in coculture and it specifically stains epithelial cancer cells (Figure 1A and ref. 21). We utilized this dye in our coculture system and developed a rapid and quantitative colorimetric assay method. In our method, the growth of cancer cells in coculture with stromal cells was determined only by measuring absorbance at 550nm (Figure 2). Since our method is quick and simple without the need to pre-label cells and transfect a marker gene, it can be applied to various tumor cells, is easily handled and suitable for screening of many samples.

Among various antitumor drugs tested, we found that 5-FU and BLM inhibited the growth of some prostate cancer cells more strongly in coculture than in monoculture (Figs. 4 and 5). Furthermore, we isolated a natural compound
from a microbial cultured broth and identified it as phthoxazolin A (Figure 5). The activation of fibroblasts is considered to be one of the approaches for suppression of tumor cell growth in a coculture (13, 14). A preliminary experiment showed that the conditioned medium from the drug-treated PrSC cells also strongly inhibited the growth of the prostate cancer cells, suggesting that the drug could activate PrSC to secrete growth suppressive factors or inhibit secretion of growth factors. Furthermore, these drugs may inhibit the action of growth factors secreted by PrSC.
It has been reported that 5-FU and BLM induce the secretion of cytokines, such as TGF-β from fibroblasts (29-31). There are several reports on the possible modulation of the tumor-stromal cell interactions by high molecular weight substances, such as HGF (32, 33) and TFG-β (34). The present results suggest that even low molecular weight compounds can influence tumor-stromal cell interactions.

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References

Figure 4. Effect of various antitumor agents on coculture of LNCaP cells and PrSC. LNCaP cells were cultured alone (○) or cocultured with PrSC (●) at 0.1% FBS with ITH in the presence of the indicated concentrations of antitumor agents. 5-FU, 5-fluorouracil; ACD, actinomycin D; ACM, aclacinomycin; ADM, adriamycin; BLM, bleomycin; CDDP, cisplatin; CPT, camptothecin; MMC, mitomycin C; MTX, methotrexate; VBL, vinblastine; VP-16, etoposide. Values are means of duplicate determinations. Each SE is less than 10%. Similar results were reproduced at least twice.

Figure 5. Differential effects of active anticancer agents on the coculture of prostate cancer cells and PrSC. LNCaP (upper), DU-145 (middle), or PC-3 (lower) cells were cultured alone (●) or cocultured with PrSC (●) at 0.1% FBS with ITH in the presence of the indicated concentrations of 5-FU (left), BLM (middle), or phthoxazolin A (right). Values are means±SD of duplicate determinations.


